# A study of immunoglobulin A biology in primary and hepatic immunoglobulin A nephropathy

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A thesis submitted for the degree of Doctor of Philosophy

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**To Yussef and Leonardo Nima**

# Abstract

IgA nephropathy (IgAN) is recognised as the commonest cause of glomerulonephritis worldwide. It is a heterogeneous condition with two major subtypes: primary and secondary IgAN, the latter being most commonly associated with liver disease. The aetiology of IgAN remains obscure. Much of the early research into its pathogenesis focused on alterations in the IgA metabolism as well as its interactions with putative food, infective, and auto-antigens. More recently abnormalities of IgA1 O-glycosylation have attracted attention as a possible key to IgA deposition. The contradictory results of IgAN research mirror its variable clinical features.

The aim of this thesis is to assess: (i) the IgA1 O-glycosylation pattern; (ii) monocyte Fc α-receptor expression; (iii) *in vitro* IgA production; and (iv) IgA isotype and sub-class profile in patients with primary and hepatic IgAN, their appropriate renal and hepatic controls, as well as normal controls.

The hinge glycopeptide of purified serum IgA1 was analysed by MALDI mass spectrometry. IgA1 was under-sialylated in all our patient groups when compared with normal controls. Patients with primary IgAN also had a small decrease in their galactose and GalNAc content as well as a number of highly abnormal species which were specific to this group.

Levels of serum and supernatant IgA from cultured peripheral blood mononuclear cells were measured using ELISA. Total serum IgA levels (but not supernatant) were raised in all four diseased groups. Serum IgA1 and IgA2 levels were both increased in primary IgAN. Polymeric IgA levels measured by size exclusion chromatography were only elevated in the cirrhosis group. There was no difference in monocyte Fcα receptor expression in any of the groups by flow cytometry.

In summary, we found significant changes in IgA1 O-glycosylation in all our patient groups and their diseased controls. These results suggest that altered glycosylation is not specific to primary IgAN. Further characterisation of the highly abnormal glycoforms specific to IgAN and their interactions is warranted. Despite elevated serum IgA levels in IgAN, we found no IgA immune system over-activity and no alterations in monocyte FcαR expression.

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## Publications arising from this thesis

E Tarelli, AC Smith, BM Hendry, SJ Challacombe, S Pouria. Human serum IgA1 is substituted with up to six O-glycans as shown by matrix assisted laser desorption ionisation-time of flight-mass spectrometry. ***Carbohydrate Research*** 2004, 339:2329-2335.

S Pouria, PH Corran, AC Smith, HW Smith, BM Hendry, SJ Challacombe, E Tarelli. High resolution profiling of O-glycopeptides derived from human serum IgA1 by MALDI-ToF mass spectrometry. ***Analytical Biochemistry***, 2004, 330:257-263.

S Pouria, E Tarelli, SJ Challacombe, BM Hendry. Analysis of IgA1 O-glycoforms in primary and hepatic IgA nephropathies. Abstract for oral and poster presentation at the 10th International Symposium of IgA nephropathy St. Etienne, France March 2004.

E Tarelli, S Pouria, PH Corran, AC Smith, HW Smith, SJ Challacombe, BM Hendry. Profiling of O-glycopeptides derived from human serum IgA1 by MALDI-ToF mass spectrometry. Abstract for poster presentation at the 10th International Symposium of IgA nephropathy as St. Etienne, France March 2004.

Helm-van Mil, AC Smith, S Pouria, E Tarelli, H Eihenboom. IgA multiple myeloma presenting with HSP associated with reduced sialylation of IgA1 ***British Journal of Haematology***, 2003, 122: 915-917.

S Pouria, E Tarelli, BM Hendry, S Challacombe. Structural analyses of IgA1 O-glycosylation by mass spectrometry in health, primary and secondary IgA nephropathies. Abstract Society for Mucosal Immunology, June 2002.

S Pouria, E Tarelli, S Challacombe, BM Hendry. Structural analysis of IgA1 hinge glycopeptide by mass spectrometry in primary and secondary IgA nephropathies. Abstract Renal Association April 2002.

S Pouria, E Tarelli, S Challacombe, BM Hendry. Analysis of IgA O-glycosylation by mass spectrometry. ***JASN*** abstract October 2001.

# List of Figures, Tables, and Abbreviations

## Figures

**Tables**

## 8.1 A summary of all the glycosylation changes found by MALDI-MS in the IgA1 HGP of the patient and control groups

### Abbreviations

| | |
|---|---|
| Ab | Antibody |
| AC | Amaranthus Caudus |
| ACE | Angiotensin Converting Enzyme |
| AMBIC | Ammonium bicarbonate |
| AS | Ammonium Sulphate |
| Asn | Asparagine |
| ASPGR | Asialoglycoprotein receptor |
| BPA | Bauhinia Purpurea |
| BSA | Bovine serum albumin |
| C of V | Co-efficient of variation |
| C | Complement |
| C1Inh | C1 esterase inhibitor |
| CAA | Caragana Arborescens |
| CD4 | Cluster of differentiation 4 |
| CD8 | Cluster of differentiation 8 |
| CD89 | Cluster of differentiation 89 |
| CF | Cystic Fibrosis |
| $C_H$ | Constant heavy chain |
| CIC | Circulating immune complex |
| $C_L$ | Constant light chain |
| Cr-EDTA | Chromium Ethylenediaminetetraacetic acid |
| CRF | Chronic renal failure |
| DTT | Di-thiothreitol |
| ECM | Extra cellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| ESRD | End-stage renal disease |
| Fab | Fragment (antigen binding) |
| FACE | Fluorophore assisted carbohydrate elecropherisis |
| Fc | Fragment (crystalline) |
| FCS | Foetal calf serum |
| FcαR | Fc alpha receptor |
| FcαR1 | Myeloid FcαR (CD89) |
| FITC | Fluoroscein isothiocyanate |
| FSc | Forward scatter |
| FSGS | Focal and segmental glomerulosclerosis |
| Fuc | Fucose |
| Gal | Galactose |
| GalNAc | N-acetylgalactosamine |
| Glc | Glucsose |
| GlcNAc | N-acetylglucosamine |
| GN | Glomerulonephritis |

| | |
|---|---|
| GS | Glomerulosclerosis |
| GTase | Glycosyltransferase |
| HAA | Helix Aspersa |
| H-chain | Heavy chain |
| HEMPAS | Hereditary erythroblastic multinuclearity associated with positive acidified serum |
| HEPES | N-[2-hydroxyethyl]-N'-[2-ethanesulphonic acid] |
| HGP | Hinge glycopeptide |
| HHL | Human hepatic lectin |
| HIgAN | Hepatic IgA nephropathy |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigens |
| HMW | High molecular weight |
| HPA | Helix Pomatia |
| HPLC | High performance liquid chromatography |
| HSP | Henoch-Schönlein Purpura |
| IC | Immune complex |
| IF | Immunofluorescence |
| IgA | Immunoglobulin A |
| IgAN | Immunoglobulin A nephropathy |
| IgD | Immunoglobulin D |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IL-1 | Interleukin-1 |
| IL-2 | Interleukin-2 |
| IL-4 | Interleukin-4 |
| IL-5 | Interleukin-5 |
| IL-6 | Interleukin-6 |
| J chain | Immunoglobulin joining chain |
| JAC | Jacalin affinity chromatography |
| kDa | Kilo-Daltons |
| L chain | Light chain |
| LMW | Low molecular weight |
| LPS | Lipopolysaccharide |
| MAb | Monoclonal antibody |
| MALDI-ToF-MS | Matrix assisted laser desorption/ionisation time of flight mass spectrometry |
| Man | Mannose |
| MCF | Median channel fluorescence |
| MHC | Major histocompatibility complex |
| MS | Mass spectrometry |
| MW | Molecular weight |
| MWU | Mann Whitney-U test |
| NANA | Neuraminic (Sialic) Acid |
| NeuGlc | N-acetylglucosamine |
| NO | Nitric oxide |
| PAF | Platelet activating factor |
| PAGE | Polyacrylamide gel electrophoresis |
| PBA | PBS/BSA/Azide |
| PBMC | Peripheral blood mononuclear cells |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PE | Phycoerythrin |
| pI | Isoelectric point |
| PIgAN | Primary IgA nephropathy |
| PMN | Polymorphonuclear cells |
| PNA | Peanut haemagglutinins |
| Pro | Proline |
| PWM | Pokeweed mitogen |
| *r* | Correlation co-efficient |
| RPE | Rubrophycoerythrin |
| RpHPLC | Reverse phase |
| SC | Secretory component |
| SDS | Sodium dodecyl sulphate |
| SEC | Size-exclusion chromatography |
| Ser | Serine |
| SLE | Systemic lupus erythomatosis |
| SS | Systemic sclerosis |
| SSc | Side scatter |
| THAP | 2,4,6-trihydroxy acetophenone-ammonium citrate matrix |
| Thre | Threonine |
| TNF-α | Tumour necrosis factor-α |
| TnPS | Thomas-Freidenrich polyagglutinability syndrome |
| UHQW | Ultra high quality water |
| UV | Ultra violet |
| $V_H$ | Variable heavy chain |
| $V_L$ | Variable light chain |
| VV | Vicia Villosa |
| WHO | World Health Organisation |
| Xyl | Xylose |

# Chapter 1: Immunoglobulin A Nephropathies

## 1.1 Introduction

The French pathologists Berger and Hinglais (1967) first described the syndrome of primary IgA nephropathy (PIgAN) in the 1960s as a glomerulonephritis characterised by mesangial IgA deposits. Since then it has been recognised as the commonest cause of glomerulonephritis (GN) in parts of the world where renal biopsies are routinely performed (Julian et al 1988). The disease accounts for 10% of patients on dialysis for end-stage renal disease (ESRD) (d'Amico 1987). Since the condition mainly affects young adults, it incurs a high cost on individuals and society.

Alcoholic liver disease is the commonest cause of secondary IgAN and manifests mainly as hepatic IgAN (HIgAN) (Van de Wiel et al 1987). When comparing their natural histories, unlike primary IgAN, HIgAN is deemed a more benign condition, having rarely been reported to progress to ESRD (Newell 1987). Mesangial IgA deposits and altered serum IgA profiles are common to both primary and secondary IgANs (Galla 1995). As such, IgA nephropathies are unique in that the diagnosis depends on the presence of IgA immune deposits on immunofluorescence (IF) studies, rather than histological appearance or a distinct clinical syndrome.

There is no effective specific treatment for IgAN and the absence of satisfactory animal models and ethical limitations to human experiments restrict our understanding of their aetiology (Feehally 1997). Hepatic IgAN, where renal and IgA system pathology co-exist, may provide a valuable model for examining the pathogenesis and determinants of disease progression in IgAN. Research aiming towards elucidation of the pathogenesis of IgAN may through consequent design of specific therapies, have a positive influence on the treatment and prognosis of this group of patients.

## 1.2 Aims of this thesis

Despite much research interest over the years into the pathogenesis of IgA nephropathies, many questions remain unanswered. None of the information we have learnt as a result of the studies I have reviewed in this thesis has led to a clear

understanding of the aetiology or brought about changes in the therapies for IgAN. The structure of the IgA1 molecule has become increasingly central to the understanding of the pathogenesis of IgAN. Alterations of the IgA1 glycosylation potentially affect all its interactions with binding sites involved in its homeostasis as well as the renal mesangium. The latter may have a crucial role in determining whether deposited IgA is cleared or sets up an inflammatory process. Glycobiology has become a burgeoning science in the diagnosis and treatment of a wide range of diseases. As such, it has the potential to make a positive impact on our understanding of the pathogenesis of IgAN and to create tailor-made therapies to prevent disease progression in IgAN.

With this background in mind we set out to investigate the features of IgA biology which we believe may hold the key to understanding the pathogenesis of IgA nephropathies. We hypothesise that abnormalities in either IgA1 O-glycosylation or IgA production and the consequent effect on the interaction between IgA and its receptors may explain the aetiology of IgAN. Furthermore, comparisons between different types of IgAN may shed light on some of the different factors that may play a role in disease progression in this heterogeneous condition. The specific aims of this project therefore are:

1. Optimal characterisation of the glycan structure of IgA1 from patients with primary and hepatic IgAN and their controls by devising an improved mass spectrometric technique.
2. To study and compare the biological characteristics of serum IgA in the patient groups and their controls.
3. To study the production of IgA using cultured peripheral blood mononuclear cells (PBMCs) from our different subject groups.
4. To assess the clearance of IgA from the circulation by studying the expression of Fcα-1 receptors (CD89) on circulating monocytes and neutrophils by flow cytometry.

## 1.3 Human IgA Biology

### 1.3.1 IgA system compartments

IgA is the most abundantly produced immunoglobulin in man with a unique heterogeneity in its molecular form and distribution (Delacroix et al 1982). It is the predominant immunoglobulin in the mucosal secretions of the body, forming the first line of defence against potentially invasive pathogens. It is also present in the serum making up 15% of the total circulating immunoglobulins (Mestecky 1988). Around 66 mg of IgA per kg body weight is synthesised each day which approximates to twice the amount of IgG and IgM produced by the body per day (Conley and Delacroix 1987). IgA exists as two isotypes, IgA1 and IgA2 (Kerr 1990). 90% of serum IgA is monomeric IgA1 produced in the bone marrow, lymph nodes, tonsils, and spleen. A small proportion of circulating IgA is dimeric and even less commonly polymeric. In contrast, secretory IgA produced at mucosal surfaces is predominantly dimeric with some IgA present in the polymeric form. The ratios of IgA1 to IgA2 vary depending on the mucosal site (Mestecky and McGhee 1987).

The mucosal and systemic IgA immune systems were initially regarded as separate compartments under separate control. In studies where either a mucosal antigen or systemic antigen were used to challenge the IgA immune system, the production of serum IgA without induction of secretory IgA and vice versa supported this notion. Although understanding the dynamics of such a system has been challenging there are suggestions that some degree of 'cross talk' may in fact occur between the two. The majority of this evidence comes from animal studies (Pabst and Reynolds 1987) although limited support has been found for trafficking of cells controlling IgA synthesis forming a kind of mucosal-marrow axis in humans (de Fitjer et al 1996). The majority of the human studies have concentrated on oral tolerance and the immune responses to immunisation via mucosal or systemic challenges with specific antigens. Such links suggest that the mucosal and systemically produced IgA whilst discrete, exist as intimately linked systems.

### 1.3.2 Structure

Human IgA is a heavily glycosylated glycoprotein which exists as a heterogeneous population of different isoforms. However, all the different forms of IgA are made of two heavy (H) $\alpha$ chains of approximately 60 kDa, and two light (L) chains (25 kDa)

producing the Fab arms and Fc region common to all immunoglobulins (Kerr 1990). Each heavy chain is folded into the N-terminal variable domain ($V_H$), followed by the constant domains Cα1 ($C_H1$), Cα2 ($C_H2$), and Cα3 ($C_H3$). IgA1 has a flexible hinge region between the Cα1 and Cα2 domains. The light chains are composed of two domains, $V_L$ and $C_L$. The code for the IgA constant heavy chain is located on the α genes on chromosome 14 (Flannagan and Rabbitts 1982). There are two α genes, $\alpha_1$ and $\alpha_2$, with $\alpha_2$ existing in two allotypic forms $\alpha_2$ m(1) and $\alpha_2$ m(2). Human IgA therefore exists as two isotypic forms, $IgA_1$ and $IgA_2$ **(Fig 1.1)**, with $IgA_2$ existing in two allotypic forms $IgA_2$ m(1) and $IgA_2$ m(2).

*1.3.2.1 IgA1 and IgA2* The two isotypes of IgA differ only in the amino acid constitution of their heavy chains, predominantly arising from the absence of 13 amino acids in IgA2 between the $C_H1$ and $C_H2$ domains. Both have N-linked glycosylation sites but IgA1 alone is distinct in having O-linked carbohydrates at its hinge region (Kerr 1990). The 13 amino acid hinge region of the $\alpha_1$-chain comprises a repeating sequence of proline, serine, and threonine residues, the latter two being variably glycosylated by carrying simple *O*-linked oligosaccharides as shown in **Figures 1.2 and 2.5**. The $\alpha_2$-chain lacks such a structure which may confer important biological advantages in rendering IgA2 resistant to cleavage by released bacterial proteases (Senior et al 1991).

The allotypes of IgA2 differ in the structure of their inter-chain disulphide bonds. A2m(1) lacks the di-sulphide bridge that connects the heavy and light chains, instead the light chains di-sulphide bond with each other via their terminal cysteines. The A2m(1) has an alpha chain which is a hybrid of A1 and A2m(2) in that its $C_H3$ is identical to that in the alpha 1 chain and the $C_H1$ and $C_H2$ are like that of A2m(2) (Kerr 1990). The biological functions of these two allotypic variants are unclear.

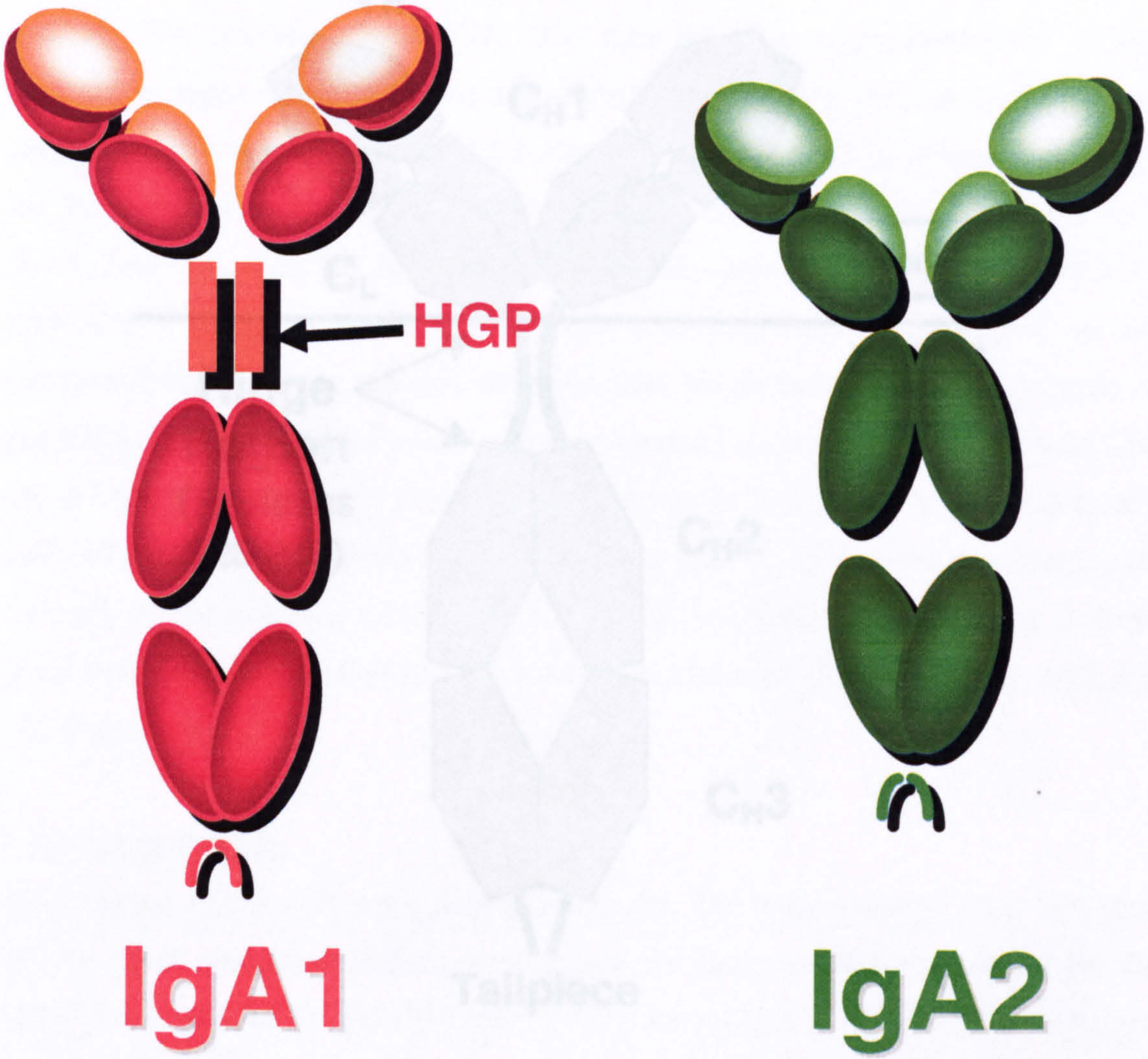



**Figure 1.1** Theoretical schemes of the monomeric IgA1 and IgA2 showing the heavy and light chains as well as the tail piece in each molecule. The human IgA1 has the additional hinge region (HGP) which is absent from both IgA2 allotypes.

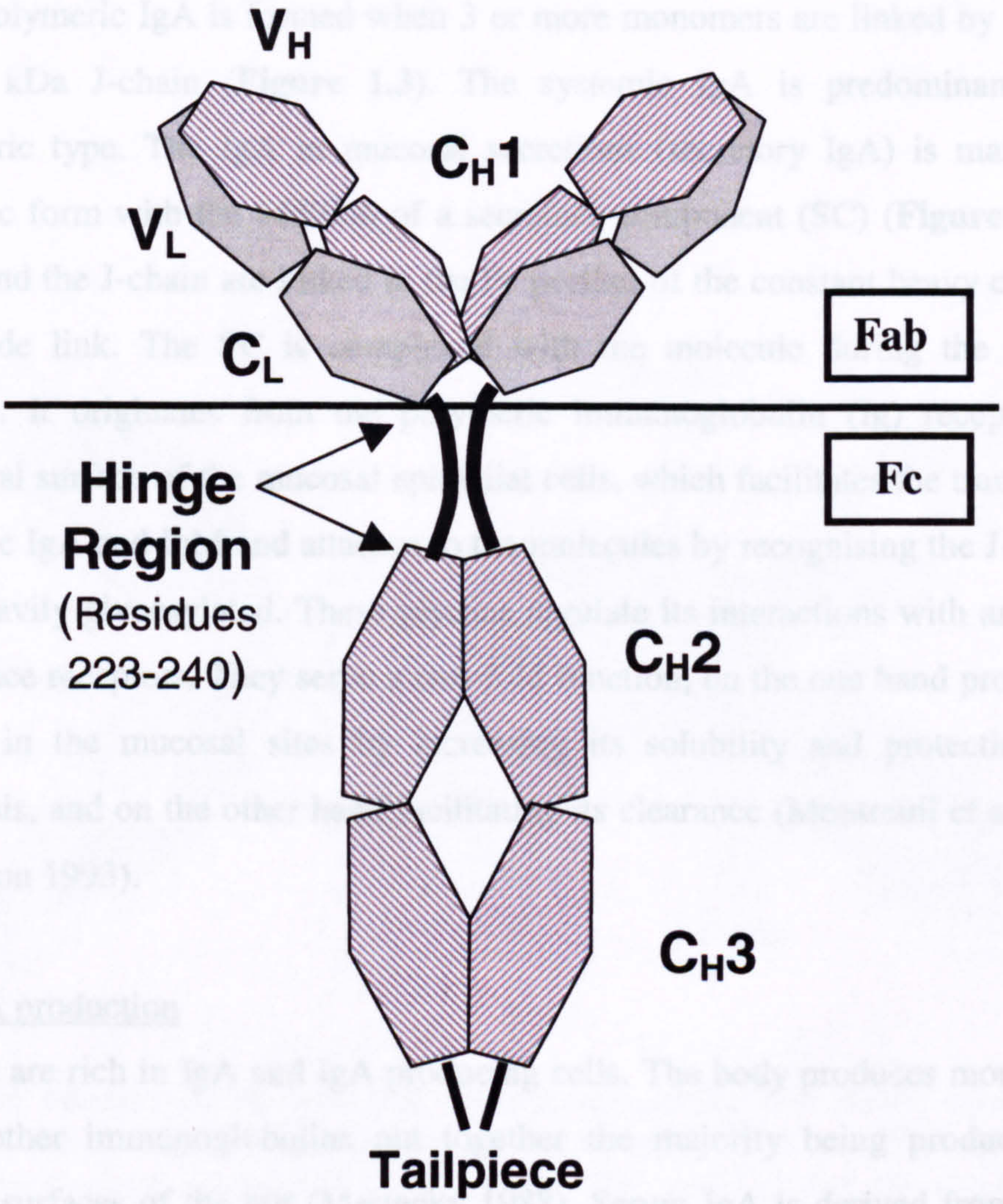



**Figure1.2** Schematic diagramme of the theoretical structure of human IgA1. The hinge region is the heavily glycosylated structure which distinguishes human IgA1 from IgA2 which is lacking in this region. Otherwise, both subtypes are formed of a pair of heavy chains (H) and light chains (L), each of which consists of one variable (V) and 3 constant (C) regions. Each molecule is divided into the Fab and Fc portions. The variable portion of the molecule is highly specific for interactions with their respective antigens and antigen presenting cells. The linear extensions or tailpieces at the C-termini of the heavy chains are also glycosylated. Image reproduced courtesy of Dr Alice Smith.

*1.3.2.2 Monomeric and polymeric IgA:* Both subtypes of IgA may be found in monomeric (molecular weight (MW) 160kDa), dimeric (MW 385kDa), or polymeric forms. Polymeric IgA is formed when 3 or more monomers are linked by a cysteine-rich 21 kDa J-chain (**Figure 1.3**). The systemic IgA is predominantly of the monomeric type. The IgA in mucosal secretions (secretory IgA) is mainly in the polymeric form with the addition of a secretory component (SC) (**Figure 1.4**). Both the SC and the J-chain are linked to the Fc portion of the constant heavy chains via a di-sulphide link. The SC is complexed with the molecule during the process of secretion. It originates from the polymeric immunoglobulin (Ig) receptor on the basolateral surface of the mucosal epithelial cells, which facilitates the transcytosis of polymeric IgA and IgM and attaches to the molecules by recognising the J-chain. The SC is heavily glycosylated. These glycans regulate its interactions with antigens and cell surface receptors. They serve a two-fold function, on the one hand prolonging its half-life in the mucosal sites by increasing its solubility and protecting it from proteolysis, and on the other hand facilitating its clearance (Montreuil et al 1982; Lis and Sharon 1993).

## 1.3.3 IgA production

Mucosae are rich in IgA and IgA producing cells. The body produces more IgA than all the other immunoglobulins put together the majority being produced by the mucosal surfaces of the gut (Mestecky 1988). Serum IgA is derived from dedicated plasma cells in the marrow that produce predominantly monomeric IgA1. By contrast, plasma cells in the mucosal areas secrete polymeric IgA1 and IgA2 which reach the mucosal fluids by translocation through the epithelial cells via the polymeric Ig receptor where they become secretory IgA. Very little of this secretory IgA enters the systemic side. Both IgA subclasses are produced at the mucosal surfaces in varying proportions depending on the site of production.

IgA production is under the control of complex cytokine pathways and regulatory lymphocytes, abnormalities of which can lead to IgA over-production, tissue deposition and damage. The control mechanisms of systemic IgA production are

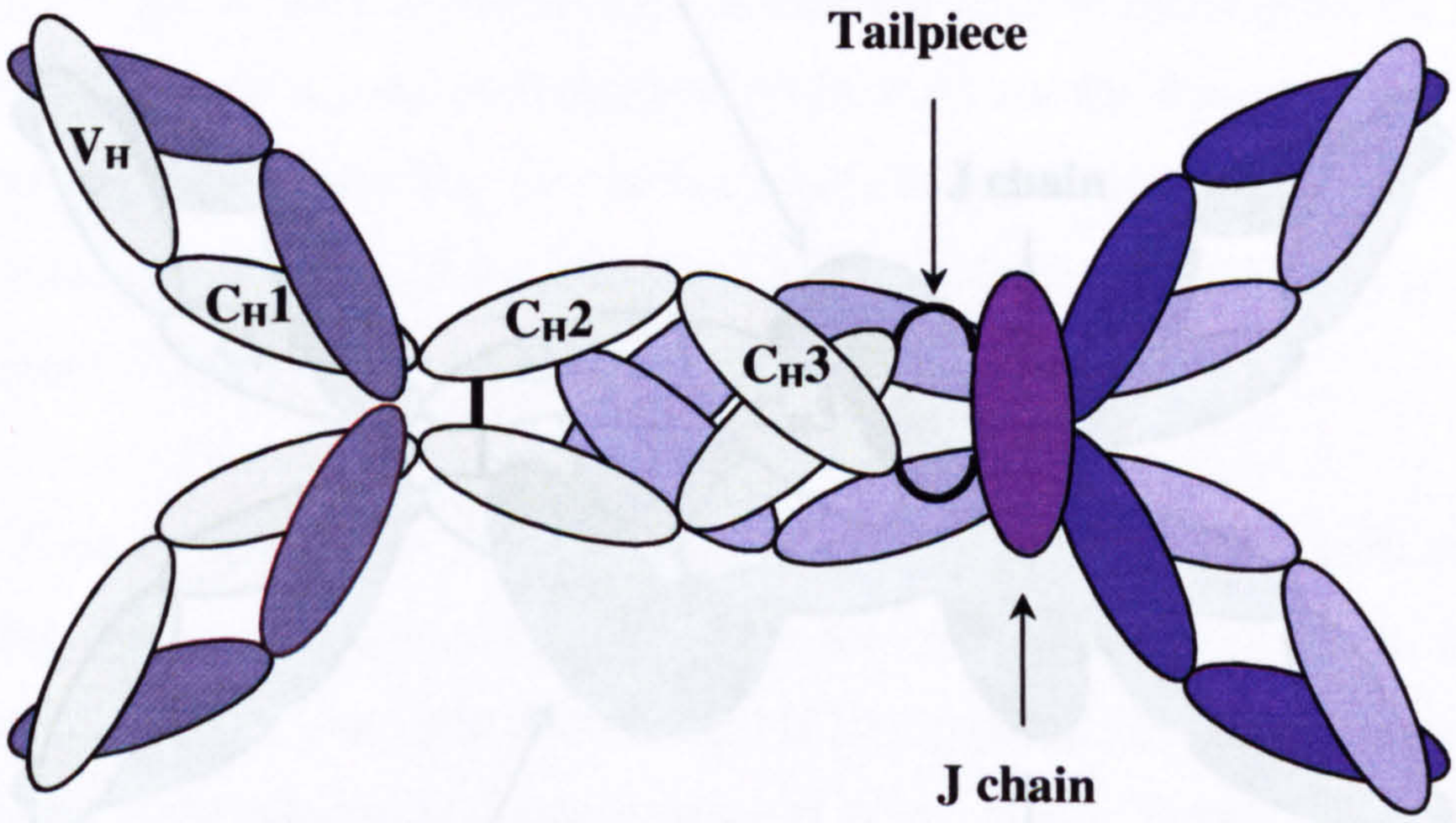



**Figure 1.3 Theoretical structure of dimeric $IgA_2$ m(1).** This model shows the Fc regions of the two IgA molecules crossed over with J chain linked to the tailpiece cysteine residues of one pair of heavy chains. The heavy chains are also linked by cysteine residues in the $C_H2$ domains to the tailpiece cysteines of the other pair of heavy chains. Image reproduced courtesy of Dr Alice Smith.

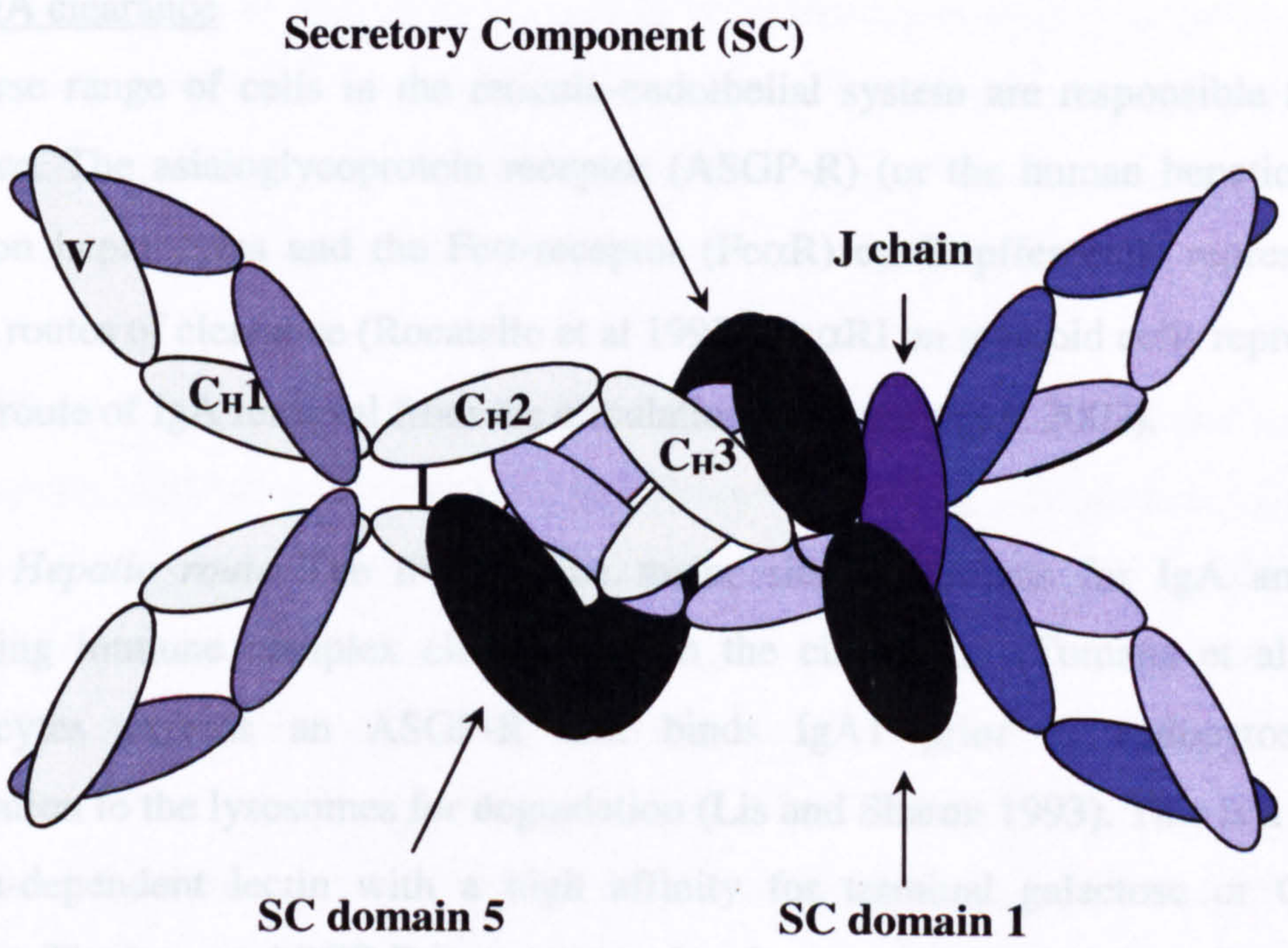



**Figure 1.4 Theoretical structure of secretory $IgA_2$ m(1).** This model shows secretory component (SC) interacting with $C_H2$ and $C_H3$ domains of both pairs of heavy chains. The fifth domain of SC is disulphide-linked to the $C_H2$ domain of one of the IgA monomers. Image reproduced courtesy of Dr Alice Smith

similar to IgG production. One study has shown $T_H2$ cells via IL-4, 5, and 6 to cause B cell class switching to IgA production (Lycke 1998). IgA production was also found to be promoted by IL-10 and TGF-β in an earlier study (Defrance et al 1992). The control of mucosal IgA production is less well understood although there is evidence for similar $T_H2$ mediated control mechanisms (Abreu-Martin and Targan 1996).

### 1.3.4 IgA clearance

A diverse range of cells in the reticulo-endothelial system are responsible for IgA clearance. The asialoglycoprotein receptor (ASGP-R) (or the human hepatic lectin) found on hepatocytes and the Fcα-receptor (FcαR) on Kupffer cells represent the hepatic routes of clearance (Rocatello et al 1993). FcαRI on myeloid cells represents a further route of IgA removal from the circulation (Monteiro et al 2002).

*1.3.4.1 Hepatic route* The liver is the major site in humans for IgA and IgA-containing immune complex clearance from the circulation (Tomana et al 1988). Hepatocytes express an ASGP-R that binds IgA1 prior to endocytosis and presentation to the lysosomes for degradation (Lis and Sharon 1993). This is a C-type, calcium-dependent lectin with a high affinity for terminal galactose or GalNAc residues. The human ASGP-R is a non-covalent hetero-oligomeric integral membrane glycoprotein with 2 sub-units. Each sub-unit is a single spanning membrane protein with one extra-cellular domain containing the C-type carbohydrate recognition domain. This domain has an amino acid sequence highly conserved for GalNAc and galactose recognition (Iobst and Drickamer 1996; Drickamer 1996; Kolatkar et al 1998). IgA1 is the principal glycoprotein cleared via this receptor. Factors favouring this route of clearance are tight clustering of GalNAc and galactose residues especially as a polymer or as part of an immune complex. IgA2 is not thought to be recognised by this receptor in humans, as the hinge region is not present in this isotype.

Kupffer cells express a receptor for the constant (Fc) portion of IgA (FcαR) which represents a further route of IgA1 and IgA2 clearance (Silvain et al 1995). Little is know about the characteristics and function of the Kupffer cell Fc-alpha receptors. It

can be postulated though, that impaired clearance via these receptors and elevated IgA levels can theoretically lead to tissue deposition, inflammation and damage.

*1.3.4.2 Myeloid cells* Circulating blood cells also express an FcαR which binds the IgA molecule. These have been detected on myeloid cells (neutrophils, monocytes, macrophages and eosinophils), erythrocytes and some types of lymphocytes. These receptors can recognise both secretory and serum IgA1, IgA2 both in the monomeric and polymeric form as well as IgA bound in immune complexes. Fc receptors for IgA are less well understood compared to Fc receptors for other immunoglobulins. The development of monoclonal antibodies against the myeloid FcαR, its characterisation and cloning has generated a higher level of interest in its characteristics.

The FcαRI is expressed on myeloid cells and is encoded by a single gene mapping to chromosome 19q13.4. It is an integral type 1 transmembrane glycoprotein of the FcR family and the Ig gene super family (Williams and Barclay 1988). It is a 55-75 kDa protein on polymorphonuclear cells and monocyte/macrophages (Monteiro et al 1990). This receptor was designated CD89 at the 5$^{th}$ Leucocyte Typing Workshop. Quantitative data suggest equal numbers of CD89 receptors on monocytes and neutrophils. Fc-alpha receptors isolated from neutrophils, monocytes and U937 cells run a similar broad band on SDS-PAGE with a molecular weight of between 50-70 kDa depending on the glycosylation. The molecules are heavily N-glycosylated (Monteiro et al 1992). A panel of monoclonal antibodies against CD89 exist: A3, A57, A62, A77, and My43 with only the latter being able to block IgA binding to FcαRI.

The direct binding of IgA via the Fc portion of the molecule to leukocytes and myeloid cell lines has been demonstrated extensively by a range of techniques in particular flow cytometry (reviewed by Kerr 1990). FcαRI has a medium affinity for IgA1 and IgA2 and binds both serum and secretory IgA well (Kerr and Woof 1999). As with the ASGP-R, IgA glycosylation can influence binding to the FcαRI, with excessively sialylated IgA having a lower binding capacity to the receptor (Basset et al 1999). After binding to its receptor, IgA can stimulate or inhibit a variety of myeloid cell functions. At the mucosal surfaces IgA cross-linking of FcαRI initiate

the myeloid cell responses such as opsonisation or phagocytosis. FcαRI-mediated responses enable the orchestration of local cytokine and inflammatory mediators action, promoting the recruitment of effector cells. The role of the FcαRI in the systemic side of the IgA immune system is not so clear. One possible role is that of IgA clearance through pinocytosis and lysosomal catabolism.

### 1.3.5 IgA Function

While the role of IgA in the mucosa as the first line of defence against pathogens is clear, the function of IgA in the maintenance of systemic immunity is less well understood.

*1.3.5.1 Serum IgA* No clear role has been ascribed to serum IgA. Evidence regarding the ability of IgA to trigger phagocytosis (Blackburn et al 1995) and cell-mediated cytotoxicity (Dunne et al 1993) has contradicted the previously held belief that IgA acted as a 'discreet housekeeper' by dampening the systemic immune response to oral and environmental antigens.

*1.3.5.2 Secretory IgA* The main role of IgA in the mucosal sites is to aggregate immobilise and consequently inhibit the adherence to and penetration of epithelial surfaces by pathogenic bacteria, viruses, and fungi (Underdown and Schiff 1986). The alternative complement pathway is also activated by aggregated secretory IgA (Lucisano Valim and Lachmann 1991). Both isotypes of IgA are capable of interacting with IgA receptors in myeloid cells at the mucosal sites, thus mediating phagocytosis, superoxide generation and the clearance of immune complexes. Other functions ascribed to SIgA are: neutralisation of biologically active antigens such as toxins, viral particles, and enzymes; enhancement of secretion of lactoferrin, peroxidase and other antibacterial factors in mucosal secretions; opsonisation for macrophages and neutrophils; and enhancing monocyte anti-bacterial activity (Mestecky 1988). Finally secretory IgA is thought to have a role in the prevention of food and environmental antigen absorption by binding and transporting them back into the lumen via the polymeric Ig receptor (Mostov and Kaetzel 1999).

## 1.4 Primary IgAN

### 1.4.1 Epidemiology

Although IgAN may develop at any age, it typically presents in young adults in the second and third decades of life and commonly in childhood and adolescence (d'Amico 1988). It is 2-3 times more common in men than in women (Schena 1990). There is marked geographical variation in the distribution of cases among and within countries. In developed countries where renal biopsies are routinely used, IgAN accounts for 30% of renal biopsies performed for the diagnosis of glomerular disease (Clarkson et al 1996). On a world-wide level, IgAN accounts for 30% of biopsy findings in Asia, 12% in Australia, 10% in Europe, and 5% in the USA (Levy and Berger 1988; Schena 1990). In North America, there are significant differences between individuals from diverse racial backgrounds. Whereas the disease is common amongst Native American Indians (35% of renal biopsies performed in that population) (Smith and Tung 1985), the white population has the same incidence as Europeans. Traditionally it has been thought that IgAN is least common (<5% of biopsy findings) in those of African extraction (Jennette et al 1985). More recently however it has been noted that IgAN is increasingly recognised in African Americans possibly due to racial admixture (Sehic et al 1997). In Africa the disease is very uncommon (Galla et al 1984). The high incidence among the Native Americans may be related to high alcohol consumption and cirrhosis rates in this population, leading to increased cases of secondary IgAN. Therefore in quoting prevalence of the disease, the presence and influence of other co-morbid conditions should be taken into account.

The quoted incidence of IgAN not only varies with geographical region but also with different screening policies and the frequency of renal biopsies. For example one explanation for the high rate of IgAN (approaching 50% of all glomerular diseases) in Japan is the vigorous urine screening of all school-aged children and their high biopsy rates (Ueda et al 1977). Autopsy and biopsy studies suggest that the quoted prevalence of IgAN is grossly underestimated. One study from Germany of 250 consecutive autopsies discovered IgA deposits in 4.8% of cases (Waldherr et al 1989). Zero-time biopsies performed at the time of renal transplants have shown that latent IgAN is a common phenomenon occurring in as many as 16% of donors (Suzuki et al 2003).

### 1.4.2 Immunogenetics

Whilst consistent genetic markers for IgAN have not been found in population studies, there is little doubt that genetic factors are involved in the pathogenesis and clinical manifestation of IgAN. This is in evidence from cases of familial IgAN (Levy 1993) and also changes in the IgA system biology in unaffected relations of patients with IgAN (Egido et al 1983a; Schena 1993). There are also reports of increased clinical renal abnormalities in relations of IgAN patients (Scolari et al 1992). A few groups have reported cases of familial clustering in IgAN although HLA associations have not been consistently identified. This may be partly due to the fact that the studies on HLA antigen associations have not had sufficient patient numbers to confer statistical power to their resutls (reviewed by Hsu et al 2000).

These findings along with the racial variations in the incidence of IgAN have prompted a search for a genetic cause in at least some of these patients. Using genome-wide linkage analyses in multigenerational families with several members with biopsy proven IgAN, Gharavi et al showed linkage of IgAN to 6q22-23 now known as the IgAN1 gene (Gharavi et al 2000.) They found 60% linkage between kindreds in a dominant model of transmission with incomplete penetrance and locus heterogeneity. Because of the high number of genes in this locus, the importance of this finding in relation to the pathogenesis of IgAN remains to be clarified. So far the analysis of polymorphic markers related to the immunoglobulin genes, MHC loci and genes identified for Fc-alpha receptors have not been found to show linkage. Further definition of the genes within the IgAN1 locus is awaited.

### 1.4.3 Clinical features of IgAN

The syndrome of IgAN may present anywhere on a spectrum between asymptomatic urinary abnormalities to a rapidly progressing glomerulonephritis (Emancipator et al 1985; d'Amico 1987). In 40-50% of cases it presents in adults in their 20s and 30s. Patients experience recurrent, self-limiting episodes of macroscopic haematuria associated with upper respiratory tract infections typically 24 hours after the onset of infective symptoms. The frequency of these episodes is higher in childhood and decreases with time (Schena 1990). Occasionally, infections of other IgA secreting tracts such as the urinary, gastrointestinal and bronchial mucus membranes may

predate the onset of haematuria. The episodes of haematuria last between 24 hours up to one week but microscopic haematuria may be persistent. Occasionally patients complain of loin pain.

About 30-40% of patients are asymptomatic, with microscopic haematuria and occasional proteinuria as the most common feature at presentation. This is often picked up at routine screening and in countries such as Japan with widespread urinary screening practices, the percentage of patients picked up with microscopic haematuria or chance proteinuria will be higher.

Some 30% of patients have a less benign onset presenting with either hypertension, nephrotic syndrome (5%), acute on chronic renal failure, or acute nephritis. Nephrotic syndrome as the initial presentation is uncommon in adults but more frequent in childhood and adolescence (Hogg 1993). Of these 10% may present with oliguria and renal failure of which a quarter may require acute renal replacement therapy (Schena 1990).

Acute renal failure at presentation is very uncommon (<5% of cases) and may be a presenting feature of a severe, rapidly progressive crescentic glomerulonephritis (Nicholls et al 1985). Alternatively it may be caused by tubular obstruction through heavy glomerular haematuria. The rest of the patients presenting with renal impairment have longstanding IgAN. Although hypertension is a common finding in all groups, it is only present in 5-10% of cases at the time of presentation (Clarkson et al 1977).

### 1.4.4 Diagnosis

The diagnosis of IgAN is a histological one based on positive mesangial IgA immunofluorescence. There are as yet no serological markers for IgAN. Serum IgA levels are elevated in a proportion of patients. In adults, serum total IgA is reported to be elevated in 30-50% and an even higher percentage of children with IgAN (d'Amico 1988). As serum IgA levels have not been measured serially or correlated with disease activity in a large population, this measurement is relatively unhelpful in either making the diagnosis or for following its course. Unlike normal serum where the $\kappa$ isotype of the IgA light chain is predominant, high $\lambda$ isotype is found in IgAN

(Lai et al1988). There are no distinctive changes in urinary immunoglobulins. Serum complement levels remain normal despite evidence of complement co-deposition in the mesangium.

1.4.5 Pathology

As with the clinical features, the histological features of IgAN fall along a wide spectrum ranging from near normal glomerular morphology, through focal and segmental proliferative changes, to crescentic glomerulonephritis (GN) (d'Amico 1987; Emancipator et al 1985). The diagnosis is defined by the presence of mesangial deposits of IgA on immunofluorescence. **Figure 1.5** shows the light microscopic and IF features of a patient with IgAN.

*1.4.5.1 Histopathology* Light microscopic changes may be minimal. The most common histological change on biopsy in about 60% of patients is glomerular matrix expansion and a diffuse and global mesangial cell proliferation confined to the mesangial compartment, without involving the capillary lumina. In some cases these changes may be focal. In 15% of cases, patients have focal endocapillary proliferation superimposed on the mesangial proliferative pattern. A further 10% have a more unusual pattern of injury including diffuse endocapillary proliferation causing occlusion of capillaries with inflammatory cells and mesangial matrix.

Crescentic changes have also been described on the background of a diffuse mesangioproliferative GN. The type of cellular infiltrates depends on the severity of the disease with increasing mononuclear cell invasion as the lesions become more advanced. One study found that the degree of renal dysfunction and the presence of crescents correlate with the number of glomerular macrophages (Arima et al 1991). Tubulointerstitial and vascular changes are no different to those found in other forms of progressive renal parenchymal disease. The histological markers associated with disease progression appear to be the presence of glomerular sclerosis, interstitial fibrosis, and vascular changes rather than proliferative changes (d'Amico 2000). These are not specific to IgAN per se.

Recently a revised grading system (H. S. Lee's grading system for IgAN) was assessed for correlation with clinical presentation and disease progression (Lee et al

2005). The grades are as follows: grade I, normal or focal mesangial cell proliferation; grade II, diffuse mesangial cell proliferation, or <25% of glomeruli with crescent (Cr)/segmental sclerosis (SS)/global sclerosis (GS); grade III, 25-49% of glomeruli with Cr/SS/GS; grade IV, 50-75% of glomeruli with Cr/SS/GS; grade V, >75% of glomeruli with Cr/SS/GS. This grading system showed good correlation with clinical features of the biopsied patients.

*1.4.5.2 Immunofluorescence* Mesangial IgA is the hallmark of IgA nephropathies. It is the sole immunoglobulin in only 15% of biopsies and co-deposited in 50-70% of cases with IgG. IgM and C3 staining (but not C1q) is also found in 31-66% of cases (Emancipator and Lamm 1989). The deposits are polymeric IgA1 and they stain more strongly for λ rather than κ light chains. The deposits are diffuse and global and may extend into the glomerular capillaries. This is associated with a worse prognosis. The deposits may be present before the appearance of any light microscopic changes and re-biopsy studies in children suggest the IgA deposits can regress whilst in clinical remission (Yoshikawa et al 1990). The intensity of IgA staining has not been found to correlate with the severity of the disease (Jennette 1988).

*1.4.5.3 Electron microscopy* Dense deposits of IgA and varying amounts of IgG, IgM and C3 are found in the mesangial and paramesangial structures. They have also been identified in the glomerular capillaries at either subendothelial, intramembranous, or sub-epithelial spaces. Other features are those of mesangiolysis presumed to be due to chronic mesangial overload by IgA deposits, glomerular basement membrane thinning, splitting, as well as the duplication of the lamina densa. These GBM changes are associated with heavy proteinuria and severe glomerular disease, such as a crescentic GN.

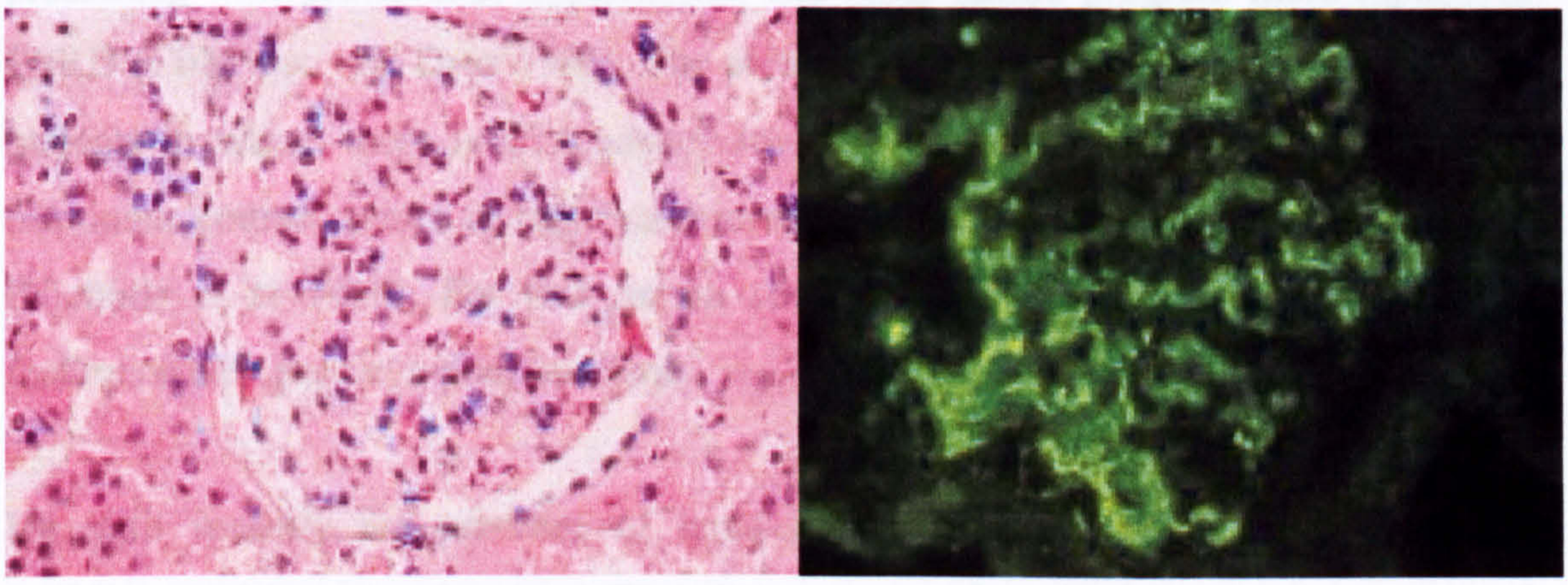


**Figure 1.5** Photomicrographs of the histological and immunofluorescence features of IgAN. The photograph on the left shows a low power image of the glomerulus in IgAN with the typical proliferative changes in a hypercellular mesangium with increased matrix deposition. The picture on the right is the image of the linear immunofluorescence pattern of IgA in the renal glomerulus of a patient with IgAN. Photomicrographs are courtesy of Dr Patrick O'Donnell.

### 1.4.6 Natural History

IgA nephropathy is thought to carry a benign prognosis for around one third of diagnosed patients who do not suffer any long-term problems. A French series which included the original Berger group of patients showed that after a follow up period of 10 years, 30% were in remission with normal renal funtion, blood pressure and complete resolution of their urinary abnormalities (Chauveau and Droz 1993). In a significant number of patients the disease progresses slowly over 20-25 years with 25-30% reaching ESRF (Clarkson et al 1984). About one third develop chronic renal failure (CRF) and its complications in particular renal hypertension. Patients presenting with proteinuria, hypertension and renal failure progress more rapidly to ESRD (Chauveau and Droz 1993). The picture is a variable one with a proportion of patients in whom there is no tendency towards progression and others in whom a fulminant course results in rapid deterioration in renal function resulting in ESRD (Nicholls et al 1985). For this reason several studies have examined prognostic features which predict a poor outcome.

Up until recently the presence of renal impairment, proteinuria of greater than 1g/24hrs, and hypertension as well as the presence of tubulo-interstitial damage and glomerular sclerosis were thought of as markers of poor prognosis. None of these factors are specific to IgAN. A study from Toronto on a large cohort of patients with long term follow up, found that mean arterial pressure and severity of proteinuria over time are the most important prognostic predictors of IgAN (Bartosik et al 2001).

A number of gene polymorphisms have also been the subject of studies in relation to disease progression in IgAN. The results of these studies have been conflicting due in part to differences in the prevalence of different polymorphisms in different populations and races (Marden et al 1995; Suzuki et al 2000). Therefore such results may not be readily extrapolated from one population to another. Nevertheless a recent study of angiotensin converting enzyme (ACE) gene polymorphisms has found that the II allele (as opposed to the ID or DD) is associated with good prognosis in IgAN, the D allele being most likely to be linked with disease progression (Syrjonen et al 2000). Other alleles such as the T235 variant of the angiotensinogen gene have been associated with disease progression through their hypertensive effects. Other gene

polymorphisms such as TNF-α, IL-1 receptor antagonist, uteroglobin, endothelial cell NO synthase, and neuropeptide Y have been studied in this context with different conclusions regarding their significance in disease progression.

Given the lack of consistency in these findings, the significance of these concepts is in doubt and far from proven. These studies have been reviewed in an article by Mustonen et al (2001). Further studies using large cohorts from various ethnic groups, followed up over a long period and with more refined methods of assessing disease activity are necessary to acquire data, which accommodate different patient cohorts in their analysis and will define those at higher risk of disease progression.

### 1.4.7 Treatment

In the absence of a clear understanding of the pathogenesis of IgAN, it has not been possible to develop treatments tailored to IgAN itself. In those with minimal proteinuria and preserved renal function, no treatment is required. In others the mainstay of therapy is aimed at controlling hypertension and proteinuria as both these complications are associated with poor outcome (Bartosik et al 2001). Immunosuppression has been used in an attempt to alter the course of the disease as well as reduce proteinuria (Laville and Alamartine 2004). Use of immunosuppressive therapies such as corticosteriods has produced inconsistent results in early trials (Julian and Barker 1993; Kobayashi et al 1986; Kobayashi et al 1988; Kobayashi et al 1996; Lai et al 1986; Shoji et al 2000; Waldo et al 1993; Welch et al 1992). This is due to small numbers of patients with different ages (including children) being employed. The cohorts studied were not all homogeneous in the severity of renal failure or degree of proteinuria. Not surprisingly these trials have not produced convincing benefits of interventions in the long-term especially in light of the very chronic nature of IgAN and its slow progress over many years. As a result, the treatment of this condition remains highly empirical in nature.

More recently a number of trials have assessed the role of steroids in the treatment of proteinuria and renal function in better-defined cohorts of IgAN patients and over longer periods of time (Pozzi et al 1999; Pozzi et al 2004). There seems to be more benefit from the use of steroids in stemming heavy proteinuria than preventing

deterioration in renal function except in the case of children with IgAN. One particular group of adults that do benefit from steroids represent the minority of patients, i.e. those presenting with nephrotic syndrome and minimal histological changes and normal creatinine. Long-term follow up has shown that the use of corticosteriods in this group does preserve renal function. When taken together, a meta-analysis of the results of various trials points to a significant effect with steroids on proteinuria and preservation of renal function (Strippoli et al 2003). This is true in non-nephrotic range proteinuria and in those patients with a relatively high risk of disease progression. The effect also seems to be most apparent early in the course of treatment. Only when sufficiently high doses are administered can this effect be sustained for up to 5 years. One must however exercise caution when drawing conclusions from meta-analyses in a heterogeneous condition such as IgAN.

Cyclosporin, cyclophosphamide, mycophenolate mofetil, azathioprine, intravenous immunoglobulin, and plasma exchange have all been used in a number of small, retrospective trials, some showing a beneficial effect on proteinuria as well as GFR over a short period. The most convincing report of a positive effect on renal outcome by cytotoxics came from Ballardie et al (2002). They showed remarkable preservation of renal function in a group of IgAN patients with a moderate risk of disease progression using prednisolone, cyclophosphamide and azathioprine. There are on-going studies assessing the efficacy of these therapies but so far most have been inconclusive.

Other treatment strategies include: (i) antigen elimination through use of antibiotics, tonsillectomies (currently being assessed in a new Japanese multi-centred randomised control trial), gluten and antigen free diets; (ii) anti-coagulation; (iii) dietary therapies such as fish oils, low protein diets, and Vitamin E supplementation; (iv) phenytoin, danazol, and dapsone. For a review of therapeutics in IgAN see Mustonen et al (2001).

ACE inhibitors and angiotensin II receptor blocking agents are increasingly used both separately and in combination. They are accepted as effective modulators of glomerular haemodynamics and as such minimize glomerular injury by effectively reducing proteinuria. One study of biopsy proven IgAN with good prognostic features

treated with enalapril versus other anti-hypertensives showed significant decline in protein excretion in the patient group as well as good preservation of renal function (Praga et al 2003). These drugs may therefore be considered as the first-line treatment in proteinuric IgAN patients with normal or slightly impaired renal function.

Finally transplantation for patients with ESRD is not only effective as a treatment but has also afforded insights into the pathogenesis of IgAN. Transplanted patients with IgAN develop mesangial deposits within months but without any overt clinical manifestations in the majority of patients. Although IgA mesangial deposits appear in 50% of patients after renal transplantation (Floege et al 1998), there had been relatively few case reports of recurrence of disease and graft loss in the literature (Berger 1988). With increasing graft survival, the number of reports of patients with recurrent disease is becoming more commonplace (Floege 2004). On the other hand, inadvertent transplantation of a kidney from an asymptomatic IgAN donor into a patient with another renal disease has resulted in the resolution of mesangial deposits in subsequent biopsies (Silva et al 1982, Cuevas et al 1987). This suggests that IgAN is not due to intrinsic renal disease, rather it is the kidney that appears to be an 'innocent bystander' in a host that is susceptible to abnormalities of the IgA1 immune system. When removed from such a pathogenic milieu, the kidney loses the features of IgAN.

## 1.5 Pathogenesis of IgAN

### 1.5.1 Introduction

The pathogenesis of IgAN is complex and involves both factors which are specific to the condition and those which are common to all renal glomerular diseases. These elements may influence the course of the condition in any of the phases of its development. The main contributing factor to the initiation of the disease process is the production of IgA with characteristics that render it more liable to deposition and triggering an inflammatory process. Secondly, the response of the mesangial cells and tissue to such deposition may be a factor in whether the condition takes hold or resolves. There may be differences between cases where the capacity to dampen inflammation in response to deposition of IgA is greater than the propensity to mount and maintain a potentially damaging inflammatory response. Thus the unfolding of the disease process may vary from individual to individual where genetic

susceptibility may affect every aspect of the pathogenesis of the condition. **Figure 1.6** summarises the interactions of factors believed to be important in the development of IgA nephropathy.

It must be said at the outset that IgAN is a morphological diagnosis and perhaps as such does not correlate to a unified clinical entity with a singular pathogenesis. The contradictory findings in the literature, the diversity of clinical presentations and outcomes make it more likely that IgAN comprises a phenotypically heterogeneous group of conditions which arise as a result of different pathogenic processes and pathways leading to the common finding of IgA deposits. Through a systematic study of these different mechanisms, we may learn about not just the pathogenesis of this group of conditions but also to revise our classification of them.

### 1.5.2 Research Models

Research into the aetiology of IgAN has been hampered by the absence of a good animal model. This has arisen for a number of reasons. Firstly there are few animal species in which the structure of IgA (in particular IgA1) is similar to the human. The IgA in rodents are more similar to IgA2 therefore rendering the study of IgA1 mesangial deposition meaningless. Rabbits have 13 different IgA isotypes also limiting their use. Most rodent models of IgAN provide information about specific antigen-antibody associated nephropathy in contrast to humans where no specific antigen for the disease has been isolated and the condition appears to be more complex than a pure immune-complex mediated disease. IgA deposits in these models are usually benign and require further manipulation to trigger renal inflammation and damage (Montinaro 1991). The compartmentalisation and handling of IgA between the systemic and mucosal systems is also distinct in humans as compared to animals. The clearance mechanisms in rodents differ from humans in that the latter lack the hepato-biliary re-circulation of IgA from the gut (Mestecky 1988).

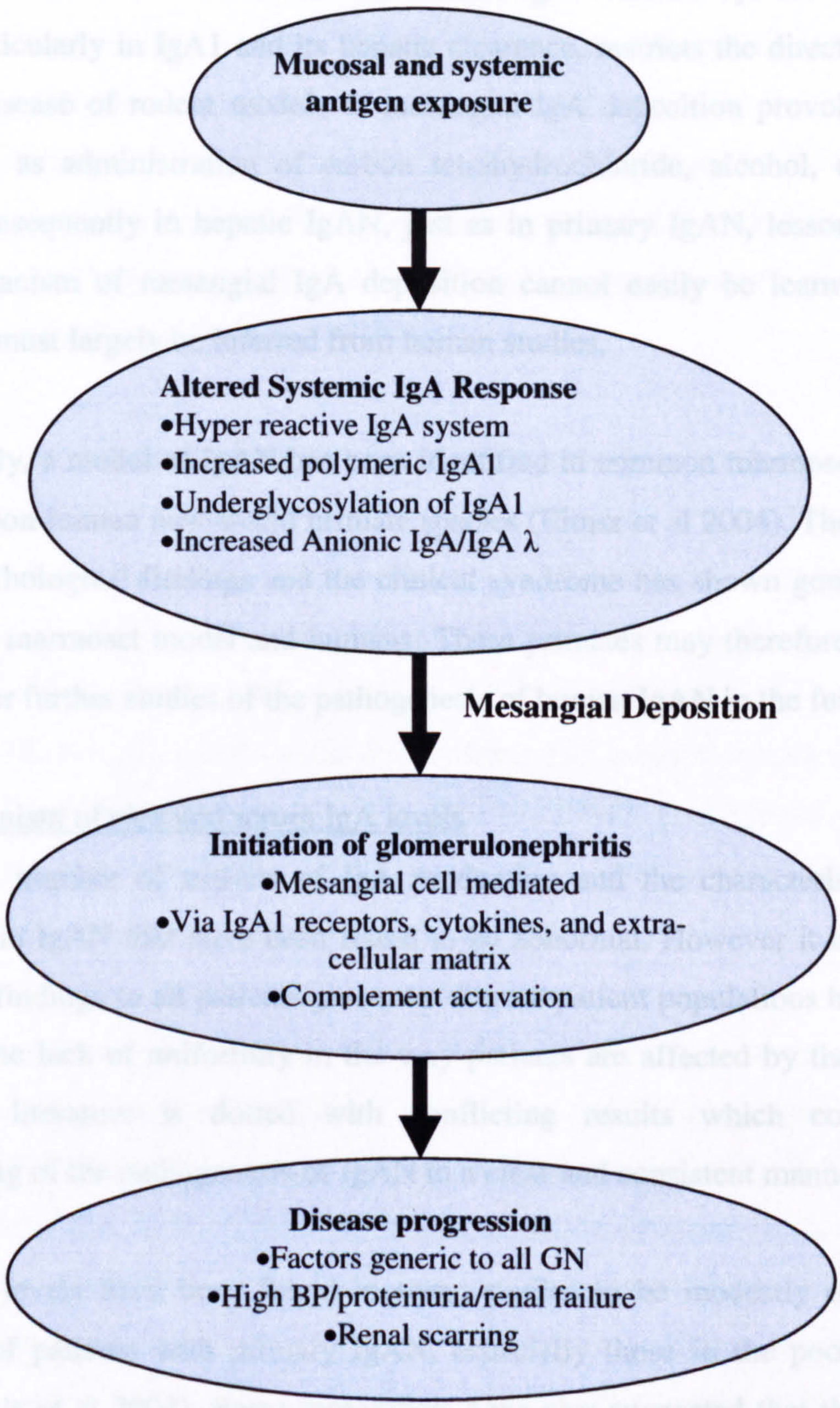



**Figure 1.6** Different factors thought to play a role in the development of IgAN. The interactions between the different elements are all modulated by the genetic predispositions of the individual. The key points in the unfolding of the disease are the preseance of IgA with altered properties. It is serum IgA with these specific characterisitics that is deposited in the renal mesangium. A small proportion of the circulating IgA and its immune complexes are prone to deposition and may also be capable of initiating glomerular injury and subsequent scarring. Failure of the mesangium to clear the deoposits adequately may result in the activation of the mesangial cells and the release of inflammatory factors. From there on the risk factors for disease progression appear to be non-specific and common to all types of glomerulonephritis (GN).

The significant differences between the human IgA immune system compared to rodents, particularly in IgA1 and its hepatic clearance, restricts the direct application to human disease of rodent models of mesangial IgA deposition provoked by liver injury, such as administration of carbon tetrahydrochloride, alcohol, or bile duct ligation. Consequently in hepatic IgAN, just as in primary IgAN, lessons about the prime mechanism of mesangial IgA deposition cannot easily be learnt from such models and must largely be inferred from human studies.

More recently, a model of IgAN has been identified in common marmosets (Callitrix jacchus), a non-human new-world primate species (Eitner et al 2004). The analysis of the renal pathological findings and the clinical syndrome has shown good homology between the marmoset model and humans. These primates may therefore represent a novel tool for further studies of the pathogenesis of human IgAN in the future.

### 1.5.3 Mechanism of elevated serum IgA levels

There are a number of aspects of IgA production and the characteristics of IgA synthesised in IgAN that have been found to be abnormal. However it is difficult to apply these findings to all patients given the diverse patient populations being studied as well as the lack of uniformity in the way patients are affected by these changes. Hence the literature is dotted with conflicting results which confound our understanding of the pathogenesis of IgAN in a clear and consistent manner.

Serum IgA levels have been found in some studies to be modestly elevated in a proportion of patients with primary IgAN, especially those in the poor prognostic group (Maeda et al 2003). Some researchers have also suggested that the polymeric form of IgA appears to be increased compared to normal and that the ratio of lambda to kappa isotypes of the light chains is also increased (Chen et al 1991). As with normals, IgA1 levels predominate over IgA2 with the IgA2 levels being normal (Feehally 1988).

*1.5.3.1 In vitro studies of IgA production:* A number of studies have found *in vitro* and *in vivo* evidence of IgA overproduction in primary IgAN (Toyabe et al 2001; Layward et al 1994; Hale et al 1986; Egido et al 1983). However other studies have

not corroborated these findings in all patients with IgAN (Linne and Wasserman 1985; Feehally et al 1986; Cagnoli et al 1985). The increased IgA production witnessed in some studies may be explained by an exaggerated antibody response to a wide array of antigens (Layward et al 1992). Many reports of dietary, environmental and infectious antigens in IgAN exist in the literature but none of these have been consistently associated with IgAN and no single pathogen has been established. The increased IgA antibodies have been found to be IgA1 by some investigators (Layward et al 1993; van den Wall Bake et al 1992).

The findings regarding the circulating polymeric and monomeric forms of IgA in IgAN have varied in different studies. A number of studies are quoted as evidence for an increased percentage of the polymeric form (Leinikki et al 1987; Layward et al 1992; Ots et al 1999). Others found no difference between the percentages of monomeric and polymeric IgA1 produced by the bone marrow of these patients compared with normal controls, while demonstrating an overall increase in both forms in the quiescent phase of the disease (Van Den Wall Bake et al 1989b). In an earlier study the same group demonstrated no increase in the serum production of polymeric IgA or IgA1 in IgAN (Van Den Wall Bake et al 1988b).

With regards to the mechanism of elevated circulating IgA levels, despite the disparities in the *in vitro* studies of IgA production, the consensus opinion is that of increased production of IgA by hyperactive circulating plasma cells. This is based on the results of a few studies showing cultured peripheral blood mononuclear cells (PBMCs) from a proportion of IgAN patients producing more IgA both spontaneously and in response to mitogenic stimulation when compared with normal subjects (Egido et al 1982; Schena 1986; Chen et al 1991). These changes were also apparent in a number of unaffected relatives of IgAN patients (Waldo 1992). Whilst some studies have produced evidence for intrinsic B cell overproduction of IgA in vitro (Egido et al 1987; Allen et al 1994; Layward et al 1994) other more recent studies of IgA production in IgAN demonstrated no significant differences in the IgA subclass synthesis when compared with controls (Baskin B et al 1996; De Fijter et al 1998). Baskin et al using in situ hybridization showed a three-fold increase in the numbers of IgA1- and IgA2-producing plasma cells, but the subclass distribution was similar to the controls. Furthermore, using the nested primer polymerase chain reaction (PCR)

for amplifying switch (Sμ/Sα) breakpoints, they demonstrated that in unstimulated PBMC the switch frequency did not differ from that of controls. In another study by de Fijter et al (1998) no evidence of IgA producing B-cell hyper-responsiveness was seen. If anything, cytokines such as IL-2 and IL-10, commonly known to induce increased IgA production in normals, failed to enhance IgA production in the patient group. They concluded that no selective IgA or IgA1 dysregulation of circulating B-cells existed and challenged the widely held paradigm that patients with primary IgAN have a hyperactive IgA immune system per se.

When viewed as a whole, the data from peripheral blood cell cultures have yielded conflicting results. This may either reflect the clinical state of the patients in a single time point or the heterogeneity of the populations under study (Feehally et al 1986). The discrepancies in the literature on the other hand may be the result of different pathogenic processes at work in different individuals within a given study cohort.

A number of other studies have focused on clarifying which cell type is responsible for the reported overproduction of IgA. Some studies have produced evidence for both intrinsic B cell overproduction of IgA *in vitro* (Egido et al 1987; Allen et al 1994; Layward et al 1994) as well as reduced T-suppressor cell while others have shown increased IgA specific T-helper cell activity (Egido et al 1983, Sakai et al 1989). One study suggested that the B-cell dysfunction described in IgAN is secondary to a primary T cell defect. Indeed when T cell specific mitogens where used in these patients and their unaffected relatives, the T-cells responded abnormally whereas the B-cells reacted normally to stimulation with LPS (Sakai et al 1988).

In another study the possible propensity towards Th2 cytokine expression by PBMCs and circulating T cells was investigated (Ebihara et al 2001). Studies of circulating lymphocytes are difficult to interpret due to the lack of certainty regarding the subsets of cells under study. The evidence regarding B and T-cell dysfunction in IgAN remains limited and indirect and may only have an aetiological role in a sub-set of patients. Unless a specific sequence of events that universally leads to the development of IgAN is identified, such a concept remains at best speculative.

*1.5.3.2 Functional studies of IgA immune system: In vivo* studies of IgA production have primarily focused on IgA response to vaccination. Exaggerated responses of polymeric IgA1 to exogenous antigens have been reported (Fortune et al 1992, Layward et al 1992). Tonsillar (Harper et al 1995) and bone marrow (Harper et al 1994b and 1995) IgA producing plasma cell numbers were increased. These studies were specific immunohistological examinations of plasma cells producing IgA, IgA1 and polymeric IgA. Conversely IgA positive B cells and production of polymeric IgA in the duodenal lamina propria is reduced with reciprocal increased IgA J chain positive B cells in the bone marrow (Harper et al 1994a). This may point to a primary defect in the mucosal IgA system possibly involving defective polymeric Ig receptor mediated transepithelial IgA transport in IgAN. One study amongst others found whilst patients with IgAN developed higher IgG levels after immunisation with tetanus toxoid, they produced lower specific IgA antibody levels in the serum, despite higher IgA production by mitogen stimulated PBMCs (Waldo 1992). Other studies of patients' post-mucosal immunisation have shown diminished IgA responses compared to controls.

More recently, researchers have examined the humoral responses to mucosal antigens in situ to avoid the use of an artificial antigen challenge. They found an exaggerated systemic IgA1 response to Helicobacter Pylori in IgAN, supporting the shift of mucosal lymphocytes to systemic sites and the subsequent production of mucosal type polymeric IgA into the systemic compartment (Barratt et al 1999).

*1.5.3.3 Antigen penetration:* IgA immune system over activity has been described as a feature of cirrhosis. However, one could speculate that this may be due to a persistent antigenaemia resulting from breakdown of the gut barrier to food and bacterial antigens. No such evidence regarding increased mucosal antigen penetration in IgAN has been found. Most (75%) IgAN patients have normal intestinal absorption studies of Cr-EDTA ruling out a generalised defect of the mucosal barrier (Jenkins et al 1988). However this does not rule out a more specific defect in immune inhibition of immune complex uptake, or a primary defect in epithelial IgA transport mechanisms (Cunningham-Rundles 1990).

*1.5.3.4 IgA clearance:* The elevated IgA levels reported in some studies of IgAN may be in part due to reduced clearance of IgA or its macromolecules by the reticulo-endothelial system. This may be due to altered expression or function of IgA receptors such as the hepatocyte ASGP-R, Kupffer cell FcαR, or of the circulating monocyte FcαRI affecting its clearance from the circulation. The liver is the main organ of serum IgA catabolism, via the asialoglycoprotein receptor and Kupffer cells. Circulating monocytes and neutrophils express an FcαRI (CD89), which binds to the IgA Fc portion before endocytosis and degradation of the molecule and its immune complexes (IC). Some studies have pointed to deficiencies in these clearance mechanisms in IgAN (Roccatello et al 1993). One study reported down regulation of CD89 expression (Grossetete et al 1998) whilst others have found that purified monomeric IgA from patients with IgAN bind less well to CD89 compared with normal controls (van Zandbergen et al 1998). The latter group have also found structural alterations in the myeloid CD89, which appears to be consistently larger, and with reduced surface sialylation than that found in normal controls.

*1.5.3.5 IgA Glycosylation:* A structural change in the IgA1 molecule such as abnormal O-glycosylation could theoretically render it less efficient as a ligand at all its various natural binding sites. In the search for IgAN specific antigens, studies of various food antigens led to the observation that IgA binding in IgAN to a number of plant derived lectins was different to normal. In 1990 Andre et al published the first study reporting altered binding of IgA from these patients to the plant lectin jacalin. This was suggestive of abnormal IgA1 O-glycosylation. Further evidence has accumulated from lectin binding, chromatography, fluorophore assisted carbohydrate electrophoresis (FACE) (Allen et al 1999), and mass spectrometry studies (Hiki Y 2001), confirming altered glycosylation in these patients.

Such structural abnormalities of IgA can alter its interactions with various immune cell receptors involved in its clearance, contributing both towards its elevated blood levels as well as rendering it more prone to deposition. Given the unique O-glycosylation of the IgA1 hinge region, it is possible that such a physical abnormality could arise from the O-linked glycans. The evidence from these studies supports the presence of under-glycosylated species of the IgA1 molecule in IgAN. Some studies

have suggested that the defect is in the loss of terminal galactose residues. None of these studies have been able to describe the stoichiometry of the IgA molecule or determine the site for glycan attachment on to the amino acid backbone. The technical challenge of producing high quality structural data has been the main obstacle to definitively addressing the issue of aberrant glycosylation in IgA nephropathies.

### 1.5.4 Origin of mesangial IgA and role of the mucosal immune system in IgAN

The source of the deposited mesangial IgA has been the subject of much discussion and controversy. Several lines of evidence suggest an involvement of the mucosal immune system in the pathogenesis of IgAN. Clinically, the acute haematuric episodes are temporally related to acute infections of the mucus membranes and intake of dietary antigens such as gluten. Furthermore, the deposited antigens in the renal mesangium that have been identified so far are either dietary components or mucosal pathogens. Impaired mucosal immunity has also been in evidence from studies of immune responses to mucosal immunisation

In addition to reports of elevated polymeric circulating IgA in IgAN, the mesangial deposits are also found to be polymeric IgA1 (Leinikki 1987, Ots et al 1999). Likewise in hepatic IgAN, most studies indicate that the deposits are polymeric IgA1 with more definite evidence for elevated polymer to monomer ratio in the serum (Newell 1987). Whilst the polymeric nature of the deposits and the concurrence of upper respiratory tract infections with acute episodes of nephritis point to a mucosal origin for the IgA, the subclass (IgA1) supports a systemic origin for the deposited molecule. This finding cannot simply be attributed to an over-spill of polymeric IgA from the mucosal system into the systemic side as the number of polymeric IgA secreting plasma cells in the mucosa are in fact found to be decreased in IgAN compared with normal controls (de Fijter JW 1996). The finding that the number of polymeric IgA producing plasma cells in the bone marrow is elevated and that systemic antigen challenge produces increased titres of circulating polymeric IgA is supportive of the hypothesis that mucosal type IgA is being produced by the bone marrow in response to both mucosal and systemic antigen challenges (van den Wall Bake AW 1988a, Harper SJ 1996). These data whilst indicative of a defect in the mucosal immune response, do not clarify the reasons for IgA mesangial deposition and the onset of inflammation.

## 1.5.5 Mechanism of deposition

The role of the normal mesangium is to maintain glomerular homeostasis. In addition to the structural and physiological roles of the mesangial cells, they have been implicated in controlling the local responses to glomerular injury. We know from autopsy studies that the mere presence of IgA deposits is not pathogenic (Waldherr et al 1989). IgA deposits are also known to regress post renal transplantation when a kidney with IgAN is transplanted inadvertently into a non-IgAN recipient. These findings suggest two things. Firstly that mere IgA deposition is not sufficient for the development of inflammation and renal injury. Secondly, that the mesangium has clearing mechanisms to remove deposited IgA. IgA deposition probably occurs when the mesangium's capacity to clear the deposits is exceeded. This may be due to a number of processes. On the one hand the sheer quantity of IgA may saturate the mesangial homeostatic mechanisms. A qualitative change in the character of the IgA may alter its interactions with mesangial components thus rendering it resistant to the mesangial clearing mechanisms. Finally a defect in the normal clearing processes employed by the mesangial cells per se may be a further hypothetical reason for IgA deposition.

It is reasonable to assume based on mounting evidence that increased serum IgA levels in themselves do not necessarily lead to mesangial deposition. Conditions such as IgA myeloma and HIV that have extremely high levels of circulating IgA are not consistently associated with IgA nephropathy. This, along with the absence of a consistent, specific mesangial or circulating antigen in IgAN makes the non-immunological interactions of IgA with mesangial extra-cellular matrix (ECM) components a more plausible mechanism for IgA deposition (Westerhuis et al 1999, Barratt et al 1999). Altered O-glycosylation of IgA1 due to defective galactosyltransferase activity may modify its molecular interactions thus promoting its deposition (Allen et al 1995, 1997).

*1.5.5.1 IgA Antigens:* Increasingly the evidence points to the fact that IgAN is not the result of a classical antigen-antibody deposition in the renal mesangium. Much effort has been put in the quest to identify such an antigen, yet no endogenous or auto-antigens have been found. Both circulating and deposited immune complexes in IgAN

have been found to contain food antigens (Russell et al 1986, Feehally et al 1987). Viral antigens too have been detected but these observations have been at best sporadic and unconfirmed, possibly not disease specific and even artefactual. Auto-reactivity to various auto-antigens have been described. These include mesangial antigens such as laminin and collagen IV as well as serum fibronectin and rheumatoid factors. However, these findings have not been confirmed as being pathogenic or disease specific. Evidence that deposited IgA is polyclonal may mean that the antigen specificity of IgA in IgAN is either not disease specific or that different antigens have variable ability to trigger IgAN in different affected individuals.

Another explanation for the immune complex formation and deposition in IgAN has come from the unusual finding that IgA itself may be the antigen against which the body produces antibodies of the IgG and IgA type. These are 'rheumatoid factors' which appear to be specifically against the under-glycosylated hinge region of the IgA1 molecule (Monteiro et al 1988; Tomana et al 1999; Kokubo et al 2000).

The precise physicochemical characteristics that make IgA pathogenic are unclear. The large size of the polymeric IgA-ICs may render them more prone to non-specific size dependent mesangial trapping and deposition (Rifai et al 2000). If production of polymeric IgA is indeed in excess of the monomer, larger IgA-ICs will predominate. These large complexes are also less likely to bind to receptors involved in their clearance and therefore become more available for deposition by trapping in the glomerular mesangium. The low efficiency of IgA to activate complement also promotes the persistence of immune complex lattice formation in the circulation. Aberrant IgA1 O-glycosylation may promote mesangial deposition given its increased tendency to self-aggregation (Kokubo et al 2000).

The charge of the IgA molecule has been the subject of investigation as a reason for enhanced tendency to deposition in the mesangium. The accepted notion of IgA as a more anionic molecule in IgA nephropathy is not a balanced reflection of the conflicting findings in the IgA literature. An early study of IgA deposits in the renal mesangium of patients with IgAN showed that the isoelectric point (pI) of eluted IgA was different to the more neutral pI of IgA obtained from normal serum The conclusion was that this more anionic form of IgA was a likely reason for increased

binding capability to the renal mesangium (Monteiro et al 1985). No renal controls were used and deposited IgA from the patient group was compared with normal serum IgA. The same group then studied serum IgA from patients with PIgAN, HSP, alcoholic liver cirrhosis, membranous GN, and systemic lupus erythematosis (Monteiro et al 1988). As no abnormal distribution of IgA isoelectric points was detected by isoelectric focusing studies, they went on to detect the charge on serum IgA using coated plates with cationic proteins. The degree of binding of IgA to these plates was taken as a measure of anionic IgA. They found increased anionic IgA in 56% of PIgAN and 40% of patients with cirrhosis. They concluded that the presence of this negatively charged IgA is involved in the aetiology of IgAN. Harada et al (1989) also showed an increase in the anionic proportion and reduced cationic proportion of serum IgA in PIgAN compared with controls in a small group of patients and controls (n=10).

Later studies however found that when the charge distribution of IgA light chains in IgA nephropathy were measured separately, IgAλ was found to be more anionic than IgAκ (Lai et al 1994, Suen et al 1997). The presence of a higher percentage of IgAλ in the circulation of patients with IgAN would explain the higher anionic/cationic ratio observed in total IgA from some patients compared with controls. Conversely, one study of the serum IgA using 2-D gel electrophoresis showed no major shift of pI values in the alpha heavy chains but the volume of the α-heavy chain bands were distributed towards the cationic region in IgAN as compared to normal healthy controls (Shuib et al 1998). They interpreted their data in support of the finding that IgA1 of IgAN patients were undersialylated.

*1.5.5.2 Mesangial components and mesangial cell receptors:* In addition to the non-specific deposition discussed above, IgA deposition may also occur in situ with mesangial cellular and matrix components. Earlier evidence for auto-reactivity of IgA to mesangial collagens has been invalidated (Coppo et al 1994). But aberrant IgA1 glycosylation appears to promote interactions with fibronectin and type IV collagen (Kokubo et al 2000). The tendency of IgA to be more anionic or cationic may also increase its interactions with mesangial proteins.

IgA deposition has not been found to relate to a specific glomerular antigen. It is not known to provoke complement activation nor is it associated with a dominant mononuclear infiltrate (except for crescentic IgAN) within the glomerulus. It is therefore possible that the glomerular injury may be a result of the interactions of the IgA molecule with the mesangium per se and not to the recruitment of other arms of the immune system. This has focused attention on the direct interaction of IgA with mesangial cells and matrix. Mesangial cells are known to bind IgA in vitro (Gomez-Guerrero et al 1993), which in turn can trigger proliferation, cytokine secretion and ECM production. Therefore, it has been postulated that they could express an IgA receptor, explaining in part the pathological features of IgAN.

Mesangial cells have been shown to express an Fcα-like receptor for IgA which is distinct from the CD89, the poly Ig receptor and the hepatic ASGPR (Moura et al 2001; Novak et al 2002). A number of researchers have studied these putative receptors. One group has defined a receptor in cultured human mesangial cells that binds IgA in an Fc alpha-dependent fashion (Barratt et al 2000). The receptor recognises both secretory and serum IgA1 and IgA2 equally but has a higher affinity for polymeric versus monomeric IgA. The receptor is immunogenically different yet shares molecular homologies with the known myeloid CD89 receptors. In a more recent study from Glasgow, primary mesangial cells from humans were found to express mRNA for a novel Fcα/μ receptor that was able to bind IgA and IgM but not IgG (McDonald et al 2002). Human transferrin receptor (CD71) expression has been recently reported on mesangial cells which may act as an IgA receptor (Moura et al 2001, Hadad et al 2003) . There is also one report on the presence of an ASGPR on the human mesangial cell (Gomez-Guerrero C et al 1998).

In summary, for IgA to deposit in the renal mesangium, the rate of deposition must exceed that of mesangial clearance. The physicochemical characteristics of the immune complexes, the antigen load, and the interaction between them and the mesangium probably govern this process. The mesangial cell is the most likely point where IgA and its immune complexes are cleared and catabolised. The evidence regarding the role of the interaction between the IgA and mesangial cells and the putative IgA receptor in the development of IgAN remains inconclusive.

### 1.5.6 Inflammation and renal damage

The mechanism of glomerular injury in IgAN is not well understood. The commonest histological feature of the disease is that of mesangial cell proliferation and extracellular matrix expansion. As the disease progresses the changes are common with any other chronic glomerulonephritis, namely glomerulosclerosis and tubulointerstitial damage.

*1.5.6.1 Mesangial cell activation:* Increasingly the evidence suggests that deposited IgA alone, without co-deposition with IgG, IgM or C3, can disrupt the balance within the normal mesangium and initiate inflammation. Mesangial IgA is thought to trigger inflammatory processes through mesangial cell and local complement activation. It may be that the presence of IgA can trigger mesangial damage via a putative Fc alpha-receptor. IgA has been shown to induce the production of TGF-β as well as extracellular matrix proteins (Lopez-Armada et al 1996). There is also evidence in the literature of IgA interactions with the mesangial cells *in vitro* initiating the secretion of a wide range of pro-inflammatory cytokines such as PAF, IL-1, IL-6, TNF-α which may then feed back into a pro-inflammatory loop causing the up regulation of the mesangial cell IgA receptors (Monteiro et al 2002).

*1.5.6.2 Cellular effectors* In addition to its interactions with the mesangial cell, deposited IgA could act as a trigger to leucocytes resident within the glomerulus. Unlike other types of proliferative glomerulonephritis, IgAN, with the exception of crescentic IgAN, is not generally associated with inflammatory infiltration of the glomerulus. Bearing in mind that 15% of resident cells in the mesangium are macrophages, which are known to express Fc alpha-receptors (Vies et al 1993) the glomerular injury may be mediated by the expansion of these cells in situ rather than through the recruitment of circulatory inflammatory cells. Macrophages may be activated in situ via their Fc alpha-receptors. One study has shown that circulating polymorphonuclear cells in IgAN not only have increased expression of Fc alpha-receptor, but also generate greater amounts of oxygen free radical species when activated by, in amongst other factors, aggregated IgA (Kashem et al 1994). It is possible that a similar process could occur in the resident or trafficking leukocytes within the mesangium contributing to the inflammatory injury.

*1.5.6.3 Complement activation:* Although the activation of complement is not necessary for the development of inflammation and renal injury in IgAN, there is evidence of some degree of local complement activation. Mesangial IgA complement activation is thought to be independent of the systemic complement cascade. Mesangial cells and podocytes may produce C3 locally via the mannose-binding lectin pathway (Matsuda et al 1998). Mesangial cells are also known to produce complement regulatory proteins and it may be that deposited IgA may modulate this immune-regulatory function (Abe et al 1998).

*1.5.6.4 Progression:* Following IgA deposition and the initiation of inflammation, the subsequent course of the disease, be it progressive damage or resolution is not specific to IgAN. The development of progressive renal failure is associated with proteinuria and hypertension which arise in tandem with vascular and tubulo-interstitial damage. These changes are also generic to all forms of glomerular disease. The mechanisms of progressive renal failure have been widely studied both in IgAN and other glomerular diseases and are not discussed further in this thesis.

## 1.6 Hepatic IgAN

### 1.6.1 Epidemiology

Hepatic IgAN is the commonest form of secondary IgAN. Other causes of secondary IgAN include suppurative lung disease and inflammatory bowel disease. IgAN has also sporadically been associated with a host of conditions ranging from connective tissue disorders such as rheumatoid arthritis, SLE, and mixed connective tissue disease; neoplastic diseases such as IgA myeloma, non-Hodgkin's lymphoma, and carcinoma of the bronchus; and infections such as HIV and schistosomiasis.

Hepatic IgAN most significantly but not exclusively occurs as a complication of alcoholic liver disease which in turn is associated with an IgA immune system disorder per se (Van de Wiel et al 1987, Hodgson 1985). Information on hepatic IgAN is based largely on autopsy and biopsy studies (Sakaguchi et al 1965, Callard et al 1975, Nochy D 1976, Berger et al 1977, Sancho et al 1982, Kalsi et al 1983, Bene et al 1988, Coppo et al 1985). Pooled autopsy and biopsy data suggest that between 50-

100% of patients with alcoholic cirrhosis have glomerular abnormalities on microscopy (Newell 1987). Where immunofluorescence (IF) has been available 30-90% of specimens stain positive for mesangial IgA deposits (Newell 1987, Axelsen et al 1995). These studies have not always taken account of viral infections, bacterial sepsis and other co-morbidities, which may be the cause of the observed glomerular injury. They have also used a wide range of criteria for biopsy. No systematic study in a large population of cirrhotics has been undertaken which would provide incidence data. Although it has been suggested that hepatic IgAN is a simple coincidence of two common conditions (Bene et al 1988), the majority of the evidence points to the existence of a distinct clinicopathological entity (Axelsen et al 1995).

### 1.6.2 Clinical Features

Hepatic IgAN is characterised by haematuria, proteinuria, elevated serum IgA levels and mesangial deposits of IgA (Kutteh et al 1982). It is usually clinically silent with mild histological changes on microscopy (Manignand et al 1981). The most common urinary abnormality, like primary IgAN, is microscopic haematuria. A small percentage of patients present with nephrotic syndrome and renal impairment (Nakamoto et al 1981). This may be more difficult to assess clinically due to hepatic hypoalbuminaemia. This same study discovered that 9.6% of cirrhotics had nephritic urine and only 1.6% were nephrotic (Nakamoto et al 1981). While the results of one study suggests that serum IgA levels (which are consistently raised) correspond with both the severity of hepatic disease and glomerular lesions (Kutteh et al 1982), the urinary abnormalities were shown to correlate with the degree of mesangial proliferation and therefore the severity of the glomerular disease in another paper (Nochy et al 1976).

Increased circulating IgA antibodies against common food and microbial antigens are reported in chronic liver disease. Circulating IgA immune complexes are elevated in up to 80% of patients with alcoholic cirrhosis and are higher in the presence of glomerulonephritis (Coppo et al 1985, Sancho et al 1981). A major proportion of these may be cryoglobulins, which are present in up to 40% of cirrhotics. There is no correlation, however, between the levels of IgA-ICs and the degree of cirrhosis.

The characteristics of IgA in hepatic and primary IgAN are summarised in **Table 1.1.** Both serum IgA1 and IgA2 levels have been found to be elevated and IgA2 is proportionally higher in some studies (Delacroix et al 1983). The different findings in the different studies may be a result of variable antibody specificity and heterogeneous cohorts of patients. The ratio of IgA1:IgA2 has not been found to be linked to the severity of the liver disease (Van de Wiel et al 1987).

In alcoholic cirrhosis, only between 25-45% of the circulating IgA is monomeric in contrast with 90% in normal individuals (Kutteh et al 1982). Once the liver damage becomes apparent, secretory IgA levels in the blood also start to rise possibly through the interruption of the normal trans-epithelial mucosal transport of polymeric IgA (Delacroix et al 1983). This may be a direct toxic effect of alcohol or other toxins. Finally, plasma C3 complement levels in these patients are depressed but it is not clear if this is due to under-production by the cirrhotic liver or increased consumption by immune complexes and cryoglobulins.

Hepatic IgAN is thought to be a benign condition that very rarely progresses to ESRD (Newell 1987). No correlation has been found between the severity of cirrhosis and the extent of renal failure. There is no established therapy for the treatment of hepatic IgAN and the prognosis depends on the course of the liver disease. There is no solid evidence that improvement in hepatic function leads to an amelioration of renal disease. However anecdotal evidence of disease regression after abstinence from alcohol, post liver transplantation or surgery for portal hypertension is cited in the literature (Babbs et al 1990). Limited re-biopsy data suggests that the glomerular morphology remains static over a number of years.

| Properties | PIgAN | HIgAN | Normal Control |
|---|---|---|---|
| **Serum IgA levels** | Elevated | Greatly elevated | Normal |
| **Serum IgA sub-class levels** | ↑IgA1 | ↑↑IgA2/↑IgA1 | IgA1 |
| **Predominant serum IgA form** | ↑Polymer | ↑↑ Polymer | Monomeric |
| **Levels of λ L-chains** | ↑ | ? | Normal |
| **IgA charge** | Anionic | ? | Normal |
| **Serum C3** | Normal | Low (?decreased synthesis) | Normal |
| **Elevated IgA-IC** | + | ++ | - |
| **Mesangial deposits** | IgA1/IgM/IgG/ C3 | IgA1/IgM/IgG/C3 | - |
| **IgA Deposit Type** | Polymeric IgA1 | Polymeric IgA1 | - |

**Table 1.1** An outline of the characteristics of serum IgA reported in the literature in both primary (PIgAN) and hepatic (HIgAN) IgA nephropathies compared with normal controls. These findings are not universal to all patients in the studies where they have been reported.

### 1.6.3 Pathology

The light microscopic features of hepatic IgAN are similar to primary IgAN. There is variable widening of mesangial matrix, thickening of capillary wall, either segmental or more commonly diffuse mesangial hypercellularity, with mesangial electron dense deposits. On immunofluorescence, mesangial IgA predominates but is often associated with lesser amounts of IgG, IgM or C3. As with primary IgAN, there may be minimal light microscopic changes despite the presence of IgA deposits on IF. Mesangial interpositioning and splitting of the glomerular basement membrane are more common than in primary IgAN. Mesangiocapillary or crescentic rapidly progressive glomerulonephritis have also occasionally been described.

Polymeric IgA1 appears to be the dominant form of deposits in the renal mesangium of patients with hepatic IgAN despite the higher levels of circulatory IgA2. Like in primary IgAN, studies of the IgA sub-class distribution in hepatic IgAN have been contradictory. This may be due to problems arising from lack of reagent specificity of the antibodies used in the studies. One early study suggested that IgA2 was the dominant deposit in hepatic IgAN (Andre et al 1980). This same study also over-estimated the levels of IgA2 deposited in primary IgAN, suggesting a possible problem with the methodology. A later study using more robust staining techniques, reporting predominantly polymeric IgA1, may therefore be more informative about the nature of the mesangial deposits (Lomax-Smith et al 1983). One recent case report of hepatic IgAN secondary to autoimmune hepatitis found mesangial deposits composed of IgA2 (Singri et al 2004). This may be due to a distinct pathological process from that of IgAN secondary to alcoholic cirrhosis.

Hepatic IgA1 deposits are a particular feature of alcoholic cirrhosis. They are present in only 12% of cases of non-alcoholic liver disease as opposed to 78% in alcoholics with cirrhosis, where IgA1 is found in a continuous pattern along the hepatic sinusoids (Burgess et al 1992).

### 1.6.4 Pathogenesis

The pathogenesis of hepatic IgAN remains obscure and a common pathogenesis with primary IgAN cannot be assumed despite the many common features that they share. Abnormalities of the IgA immune system, antigen overload, defective liver clearance

of IgA and cellular control of IgA production may influence the distribution and behaviour of IgA in liver disease.

*1.6.4.1 Increased polymeric IgA production* Raised circulating IgA and IgA immune complexes may represent an appropriate response of a normal IgA immune system to excess antigen exposure and persistent antigenaemia resulting from diminished mucosal integrity. There is also some evidence of an exaggerated intrinsic IgA system activity. In alcoholic cirrhosis circulating B-cells show increased spontaneous and mitogen stimulated production of IgA *in vitro* possibly due to abnormal T cell cytokine patterns or suppressor cell function (Giron et al 1992). Peripheral blood mononuclear cells also have an enhanced IL-6 response leading to further IgA secretion from B-cells and thus may promote an auto-amplification loop *in vivo* (Deviere et al 1999).

*1.6.4.2 Decreased clearance* The binding of IgA to its hepatic receptors may be impaired in hepatic IgAN thus reducing its clearance rate. The fractional catabolism of IgA and its complexes is reduced in alcoholic cirrhosis (Delacroix et al 1983) and all hepatic removal routes for these complexes are impaired (Roccatello et al 1993). This reduced clearance may result from alterations in the structure (and therefore interaction) of the receptor, the IgA molecule itself or the surface distribution of the receptor. In health the ASGP-R is based in the sinusoidal/lateral aspect of the hepatocytes. In cirrhosis, loss of hepatic polarity may account for the observation that the receptor reverts to the canalicular surface, thus preventing exposure of the receptor to circulating IgA and the IgA immune complexes (Burgess et al 1992). There is also evidence that the expression of Fc-alpha receptors in cirrhosis is reduced on circulating monocytes and that the endocytosis of the immune complexes of IgA is defective (Silvain et al 1995). This may in turn contribute to reduced clearance of IgA from the circulation. These findings may not be directly applicable to hepatic IgAN and such studies are yet to be performed in this group of patients.

*1.6.4.3 IgA mesangial deposition* There have been no direct studies of the mechanism of mesangial IgA deposition in hepatic IgAN. High levels of food and bacterial antigens circulating in cirrhotic patients may lead to formation of antigen-antibody complexes either in situ in the glomerulus or in the circulation. But this presumption

has not been confirmed and indeed there are no studies to reliably identify the antigens within the mesangial deposits. As in primary IgAN, abnormal glycosylation may modify interactions influencing mesangial deposition and glomerular injury. These have not been studied in hepatic IgAN. The explanations for variable glomerular injury and disease progression in hepatic IgAN are no clearer than in primary IgAN. Further studies of the pathogenesis of hepatic IgAN may provide valuable insights into IgA pathobiology and the range of processes involved in IgA tissue deposition in both primary and secondary IgANs.

## 1.7 Summary

Despite the identification of a whole variety of immune and non-immunological processes in idiopathic IgAN, the basic pathogenic mechanisms involved in this condition remain obscure. We still do not know exactly why IgA deposits in the renal mesangium and why in some patients it produces inflammation and glomerular injury whilst remaining an innocuous feature in others. Patients with hepatic IgAN can be studied in a comparative manner alongside patients with primary IgAN with the aim of elucidating the different mechanisms involved in the development of this heterogeneous condition.

# Chapter 2: IgA Glycosylation

## 2.1 Introduction

Protein glycosylation is a common form of post-translational modification. It plays a crucial role in the structure, function, and interactions of glycoproteins. Glycobiology is a relatively young science, studying the synthesis, structure and function of the sugar residues on glycoproteins and the control mechanisms involved in the glycosylation process. As a discipline it has been overshadowed in the past by the interest in the biological and pathological role of proteins. However, it has gained increasing relevance over the last two decades, as more diseases have been associated with abnormalities of protein glycosylation. Glycobiology has become more prominent as a discipline in investigating the pathogenesis of IgA nephropathy over the last 15 years. The absence of a consistent antigen as a trigger for IgA deposition prompted the search for a physicochemical property in the IgA molecule itself which would render it more liable to deposition. In this chapter the background to my IgA glycosylation work will be reviewed.

## 2.2 Protein Glycosylation

Glycoproteins are formed when specific amino acid residues on the protein backbone are linked covalently to carbohydrate chains. The presence of these chains creates significant heterogeneity in the structure of the glycoproteins. This is due to the multiple possible combinations of monosaccharides and their diverse linkage to various amino acid sequences. Furthermore, each glycosylation site on a protein may have variable occupancy with different glycans giving rise to a multitude of glycoforms of a single glycoprotein (Lis and Sharon 1993).

### 2.2.1 Glycans present in glycoproteins

Two different types of monosaccharides occur in mammalian glycoproteins. Those constituted from a 6-carbon ring are Glucose (Glc), Mannose (Man), Galactose (Gal), Xylose (Xyl), Fucose (Fuc), N-acetyl glucosamine (GlcNAc), and N-acetyl galactosamine (GalNAc). These monosaccharides predominantly form the core sugars in the glycan chains of glycoproteins. Various forms of a 9-carbon sialic acid residue are also found in glycoproteins of which N-acetyl neuraminic acid (NeuNAc) and N-

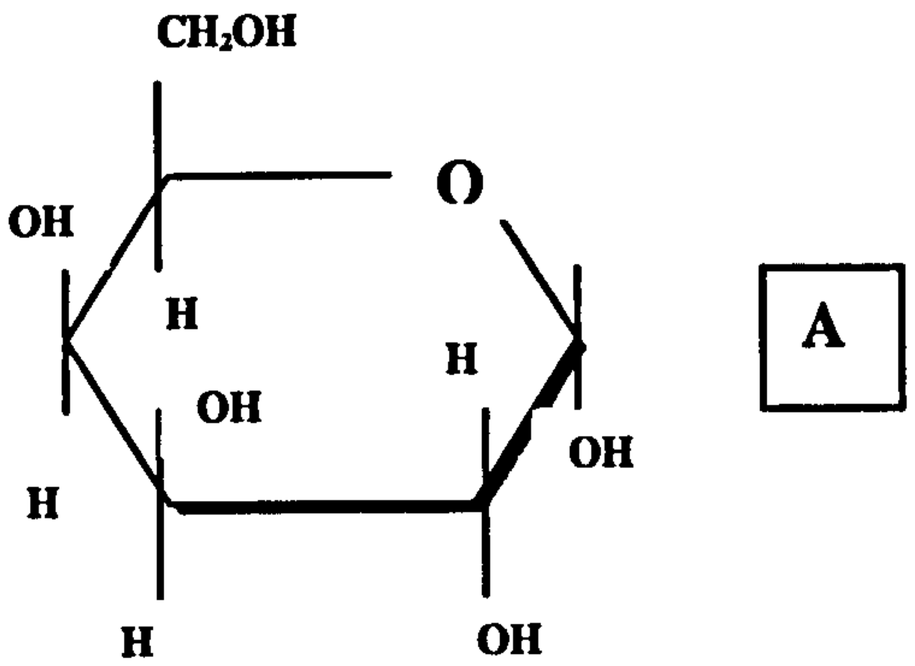
glycolyl neuraminic acid (NeuGlc) are the commonest (**Figure 2.1**). These glycans are different to other monosaccharides found in mammalian glycoproteins in a number of respects. The commonest type of sialic acid found in man is N-acetyl neuraminic acid (NeuNAc) with an acetyl group at the 5-carbon position. These sugars usually take on the terminal position of the N- and O-glycans and as such play an important role biologically, being highly negatively charged, reactive species at the outer surface of the glycan core. The labile nature of sialic acids means that they are readily lost during the life span of a protein. This is particularly true as a protein ages and under experimental conditions, and differences in sialylation may simply reflect the degradation of the glycoprotein both *in vivo* and *in vitro* (Varki 1992). This can confound investigations of protein sialylation and has led to investigators focusing on the core glycans rather than on the sialic acids.

2.2.2 Glycan linkage to protein back bone

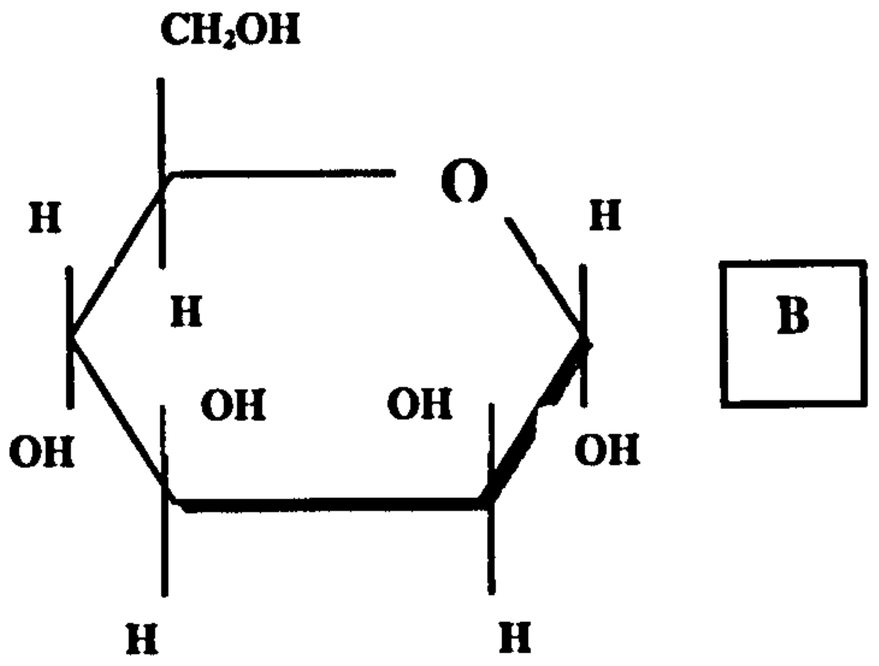
Glycans are linked covalently to the protein backbone in two different ways, via an N-link to asparagine residues or through an O-link to serine or threonine (Montreuil et al 1995).

*2.2.2.1 N-links* N-linked glycosylation is common in both secreted and membrane bound proteins and is a feature of immunoglobulins. The N-linked sugars are connected to the protein chain via GlcNAc in a α1-N linkage with an asparagine residue as a co-translational event in the endoplasmic reticulum (Opdenakker et al 1993). N-linked oligosaccharides have a common inner core structure of 5 sugars which is conserved in all N-glycopeptides. The remaining sugars exist in a number of branched chain varieties (**Figures 2.2a-c**). The high mannose type is formed of mannose and GlcNAc residues alone. The complex type contains the latter residues as well as galactose, fucose and sialic acid. The hybrid type is a mixture of the latter two types (Montreuil1984).

*2.2.2.2 O-linked glycans* O-linked glycoproteins have a range of 1-20 sugars which are found clustered in heavily glycosylated domains. These are found in high concentration predominantly in membrane bound proteins. O-glycans are rarely found in serum proteins, the exceptions being IgA1, IgD, C1 esterase inhibitor, chorionic




A

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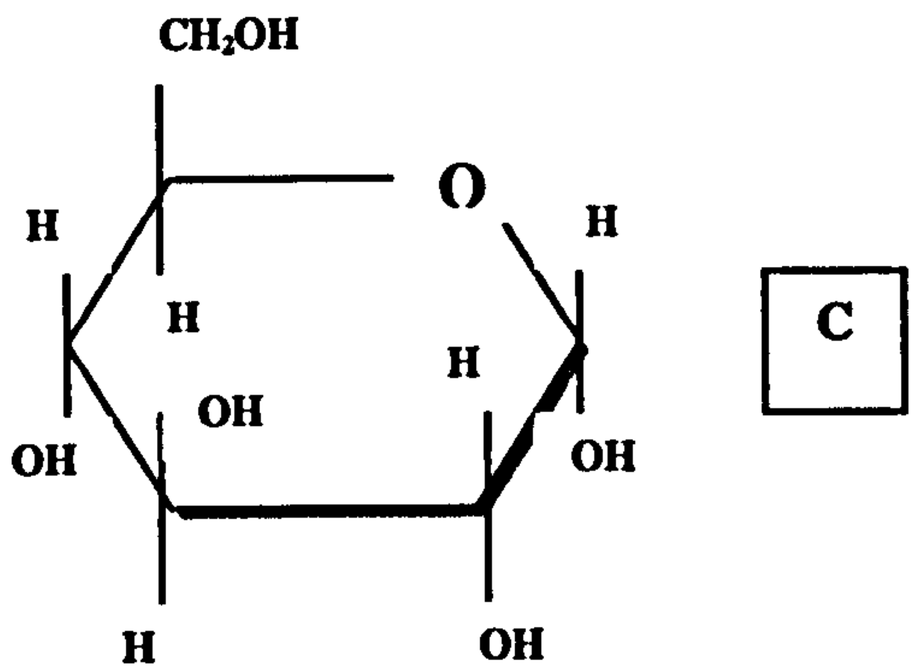


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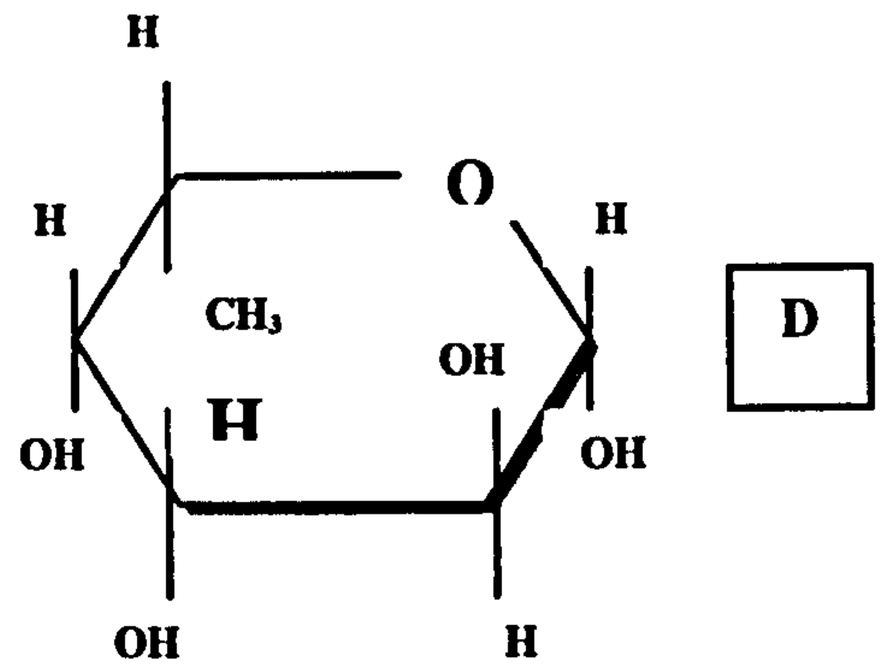


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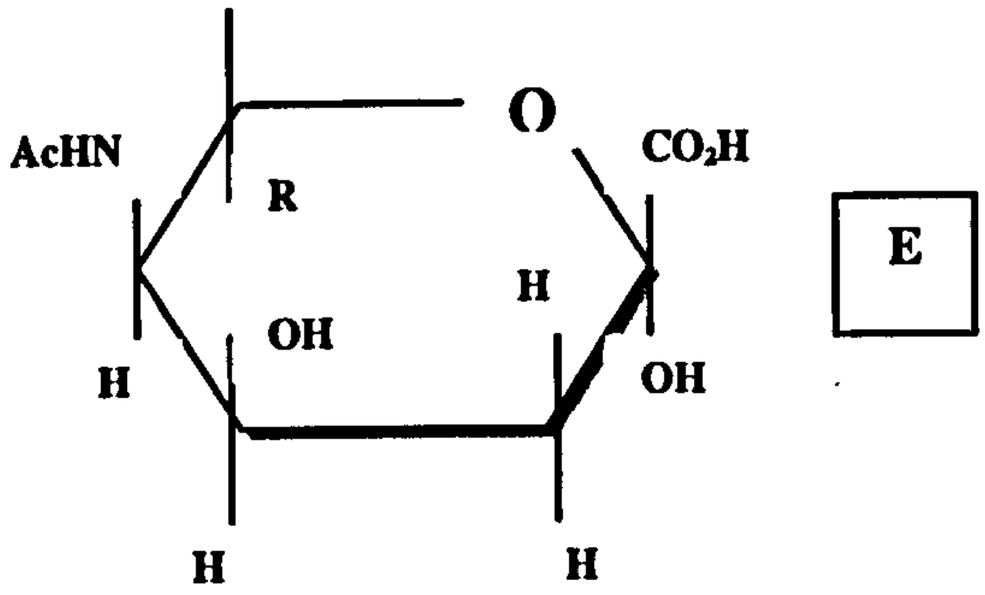


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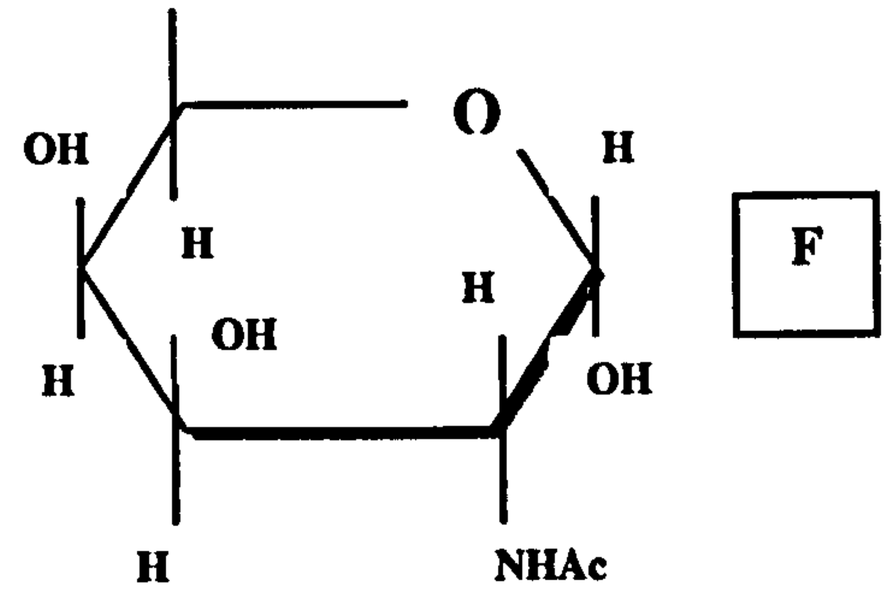
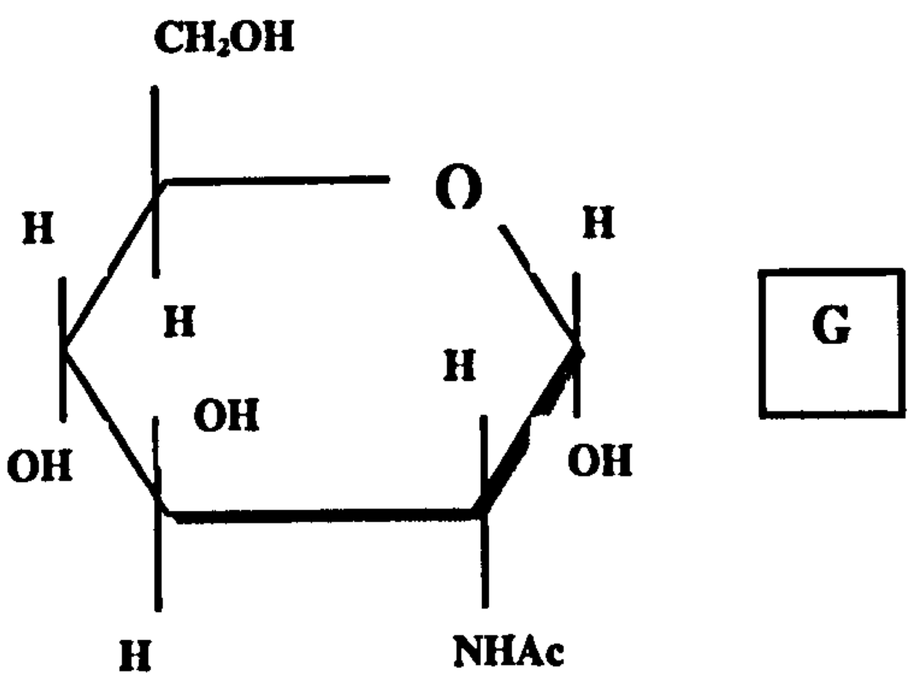



F




G

**Figure 2.1** The structure of some of the monosaccharides present in mammalian glycoproteins is demonstrated: A. Galactose ($C_6H_{12}O_6$), B. Mannose, C. Glucose, D. Fucose, E. Sialic Acid, F. GalNAc, G. GlcNAc.

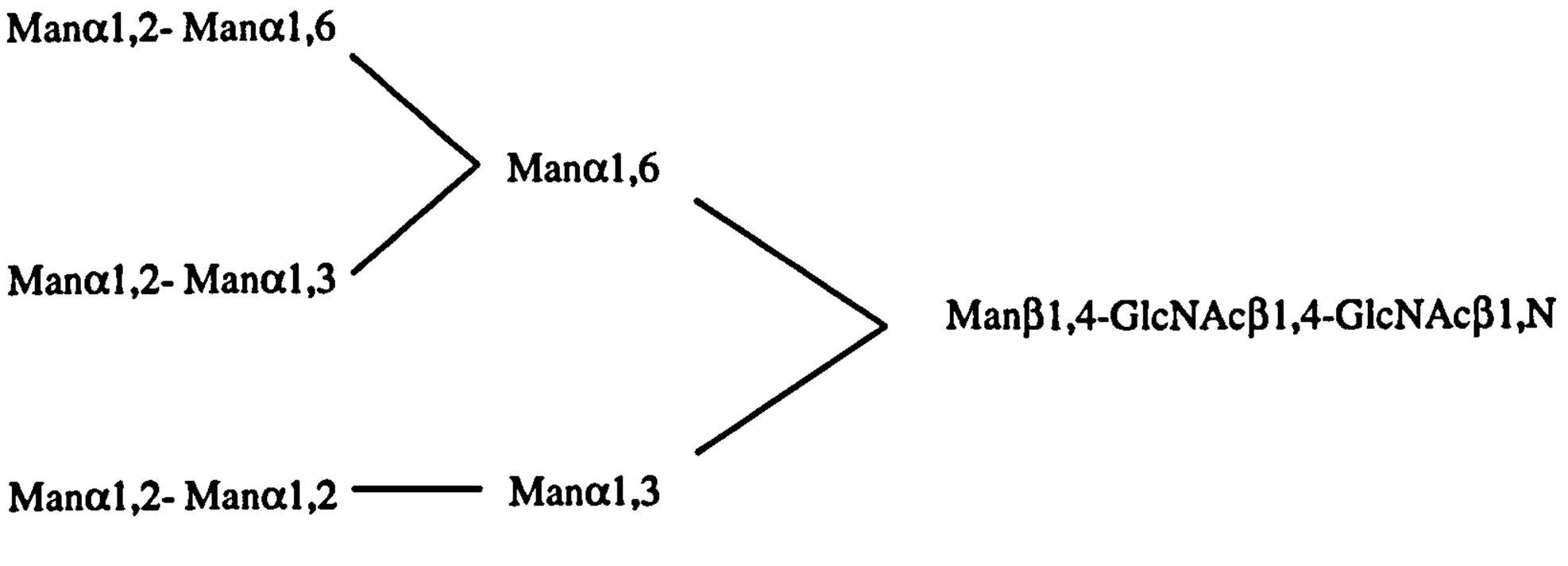



**Figure 2.2a** showing high mannose type side chains of N-linked glycans

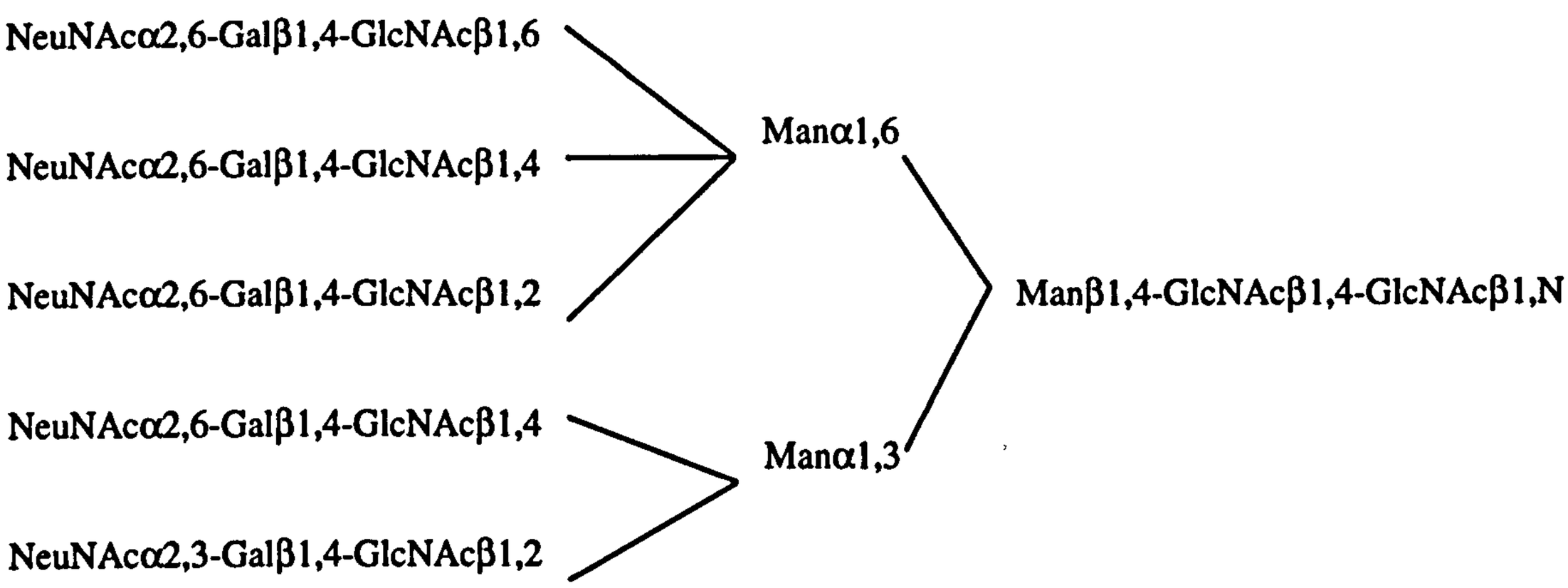



**Figure 2.2b** showing complex type side chains of N-linked glycans

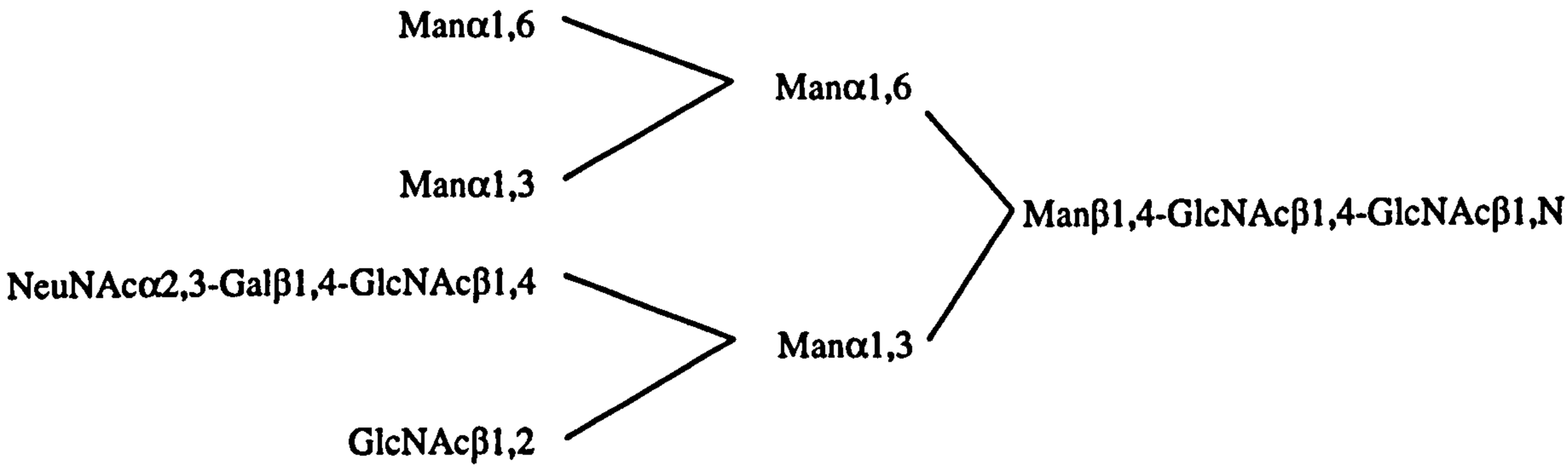



**Figure 2.2c** showing hybrid type side chains of N-linked glycans

gonadotrophins, erythropoetin, plasminogen, fetuin, and a number of cytokines such as IL-2 and IL-6 (Hortin and Trimpe 1990).

O-linked glycosylation typically occurs on repeating sequences of amino acids which are rich in serine, threonine, and proline as a post-translational modification in the Golgi apparatus. The glycans are added sequentially by glycosyltransferases to the serine and threonine residues. The tertiary structure of the complete protein does not allow glycosyltransferases to access all potential glycosylation sites. The presence of this repeating sequence of amino acids tends to form an extended structure with many β turns rather than conforming to α helix formation. Furthermore, clusters of O-linked glycans on such peptides create a rigid structure, allowing the creation of long mucin domains (Hounsell and Davies 1993, Lis and Sharon 1993).

There are three types of O-linked glycans (Montreuil 1984). The mucin type sugars are based upon a 1,3 GalNAc connection to a serine or threonine residue. Further substitution with simple monosaccharides such as galactose or neuraminic acid can occur in a single chain. This is the type found in immunoglobulins and in the IgA1 hinge region (**figure 2.3**). The proteoglycan type is based on a xylose residue attached via a 1,3 serine residue creating a long single chain of repeating disaccharides. The collagen type is made up of a galactose in a 1,5 linkage with hydroxy-proline or hydroxy-lysine.

2.2.3 Protein glycosylation enzymes

*2.2.3.1 Glycosyltransferases* Diverse and numerous but poorly characterised glycosyltransferases (GTase) and glycosidases are responsible for protein glycosylation (Kleene and Berger 1993; Narimatsu 1994). Most of the glycosylation reactions that generate the great diversity of oligosaccharide structures of eukaryotic cells occur in the Golgi apparatus (Kleene and Berger 1993). Glycosylation pathways may also occur in the cytosol and the endoplasmic reticulum (Montreuil et al 1996). More recently further information regarding the functional organization of Golgi-resident glycosyltransferases as well as X-ray crystal structure determination of glycosyltransferases has become available, shedding light on the molecular basis for donor and acceptor substrate specificities as well as GTase catalytic function (Breton et al 2001).

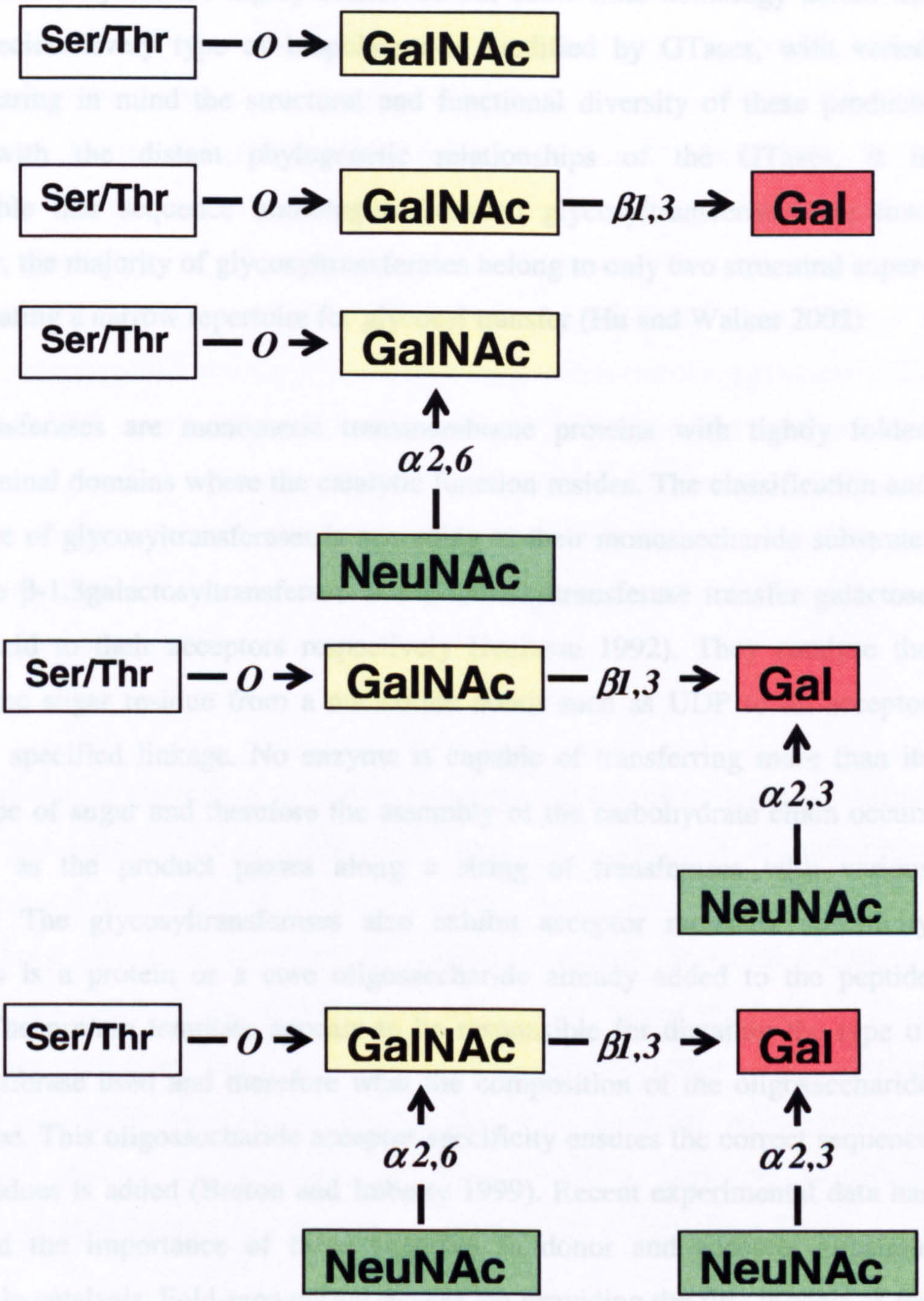



**Figure2.3** The various permutations of the IgA1 hinge glycopeptide O-glycosylation. Each serine or threonine residue in the amino acid backbone can potentially be substituted with a GalNAc residue via an α1 O-link. This can either be a terminal GalNAc as in example 1, or it may be further substituted with a terminal galactose residue via a β1,3 link as in example 2. The terminal GalNAc may also be directly substituted by a neuraminic acid residue via an α2,6 link as shown in permutations 3 and 5. Galactose residues may also have a terminal sialic acid residue linked to them via α2,3 links as shown in examples 4 and 5.

Functionally, glycosyltransferases are one of the most diverse group of enzymes. The genes for these enzymes are highly conserved but show little homology across the different species. Every type of biopolymer is modified by GTases, with varied effects. Bearing in mind the structural and functional diversity of these products combined with the distant phylogenetic relationships of the GTases, it is understandable that sequence homologies between glycosyltransferases are low. Interestingly, the majority of glycosyltransferases belong to only two structural super-families, creating a narrow repertoire for glycosyl transfer (Hu and Walker 2002).

Glycosyltransferases are monomeric transmembrane proteins with tightly folded carboxy-terminal domains where the catalytic function resides. The classification and nomenclature of glycosyltransferases is according to their monosaccharide substrate. For example β-1,3galactosyltransferase and α-2,6sialyltransferase transfer galactose and sialic acid to their acceptors respectively (Joziasse 1992). They catalyse the transfer of the sugar residue from a nucleotide donor such as UDP to an acceptor protein in a specified linkage. No enzyme is capable of transferring more than its specified type of sugar and therefore the assembly of the carbohydrate chain occurs sequentially as the product passes along a string of transferases with various specificities. The glycosyltransferases also exhibit acceptor molecule specificity whether this is a protein or a core oligosaccharide already added to the peptide backbone. The protein template appears to be responsible for dictating the type of glycosyltransferase used and therefore what the composition of the oligosaccharide chains will be. This oligosaccharide acceptor specificity ensures the correct sequence of sugar residues is added (Breton and Imberty 1999). Recent experimental data has demonstrated the importance of these enzymes in donor and acceptor substrate binding and in catalysis. Fold-recognition studies are providing the first models of the catalytic domains of some of these enzymes (Breton and Imberty 1999).

O-glycan synthesis is by far simpler than the pathways involved in N-oligosaccharide production. It begins in the cis Golgi apparatus with the transfer of the first sugar residue GalNAc from a nucleotide sugar by a specific polypeptide O-GalNAc transferase. The chain then grows by the addition of further Gal, Fuc, or GlcNAc residues in the medial Golgi. Sialylation occurs in the trans Golgi (Brockhausen 1995).

N-oligosaccharides biosynthesis starts in the endoplasmic reticulum ER with a large precursor oligosaccharide that contains 14 sugar residues. This precursor is linked to dolichol pyrophosphate, which acts as a carrier for the oligosaccharide which is then transferred to an Asn residue on the growing polypeptide chain (Verbert 1995). It is at this point that all the glucose and mannose residues are removed by specific glycosidases, producing an oligosaccharide with 10 residues instead of 14. The maturation of the N-oligosaccharides takes place in the Golgi complex via a coordinated and sequential set of enzymatic reactions, which remove and add specific sugar residues. The enzymes involved (glycosidases and glycosyltransferases) are located in the cis, medial, and trans Golgi (Schachter 1995). The reaction product of one enzyme is the substrate for the next. The high-mannose and hybrid oligosaccharides appear as intermediates along the processing pathway. The complex type is the mature form of N-linked oligosaccharides.

*2.2.3.2 Regulation of glycosylation* Factors regulating glycosyltransferase expression are not well understood. They are often highly tissue and cell specific and may occur at precise stages of differentiation. The control levels appear to be at the transcription level given that protein levels correlate with mRNA expression (Kleene R and Berger 1993). Cytokines and bacterial products may be involved in the regulation of their function. It has been postulated that increased IL-6 secretion triggered by infection may lead B-cell differentiation into plasma cells with increased sialyltransferase activity at the expense of galactosyl transferase function. Likewise genetically induced changes in GTase activity may affect glycosylation of IgA (Shur 1994).

## 2.2.4 Micro-heterogeneity of glycans

The mixture of glycosylated variants is known as its glycoforms where the same peptide sequence exists with more than one oligosaccharide at the same glycosylation site. In fact a single glycan may occur on the same glycosylation site but in different combinations with other glycans and via different linkages. A single glycopeptide may have a different structure in part due to the folding of the peptide moiety and its exposure to and recognition by glycosyltransferases as a potential oligosaccharide

acceptor. The prevalence of glycoforms also depend on the cell type in which it is found as well as its enzymatic machinery, its stage of development and the nutritional or pathological state.

## 2.3 IgA Glycosylation

### 2.3.1 IgA1

Both subclasses of IgA, like all immunoglobulins, are glycosylated. Both IgA1 and the two allotypes of IgA2, have N-linked glycosylation sites. IgA1 is heavily glycosylated with around 6-7% of the molecule made up of carbohydrates. There exist two N-linked sites on human IgA1 ($Asn^{263}$ and $Asn^{459}$) that carry complex type residues. N-glycans of normal human IgA1 are heavily galactosylated and sialylated with a large degree of heterogeneity as well as carrying a number of mannose, N-acetylglucosamine and fucose residues (Field et al 1994).

In addition to these N-linked glycans, IgA1 is also O-glycosylated in the hinge region connecting the CH1 and CH2 domains (Kerr 1990) as shown in **Figure 2.4.** Although the sequence of IgA2 differs only by 20 amino acids compared with IgA1, the highly unusual structure of the hinge region makes it unique in its glycan and protein composition. Humans are the only species with such an unusual hinge region structure. The presence of O-linked GalNAc, Gal and NeuNAc residues is a rare phenomenon in circulating serum glycoproteins and is the feature which also distinguishes IgA1 from IgA2 (Field et al 1989). This feature is responsible for the IgA1 molecule's susceptibility to bacterial proteases. There is much variety in the glycoforms present in IgA1 due to the multitude of glycosylation sites and glycan number and position variations. The presence of many closely packed O-linked sugars makes the structure of the IgA1 hinge region distinctive.

Much of the early information about IgA1 structure and glycosylation came from studies of myeloma IgA (Baenzinger and Kornfeld 1974). These showed that the hinge glycopeptide is composed of a 21 amino acid sequence with repeating serine, proline and threonine residues and that the sites of O-glycosylation were located on 5 serine residues. The first studies of the normal IgA1 glycan structure were performed

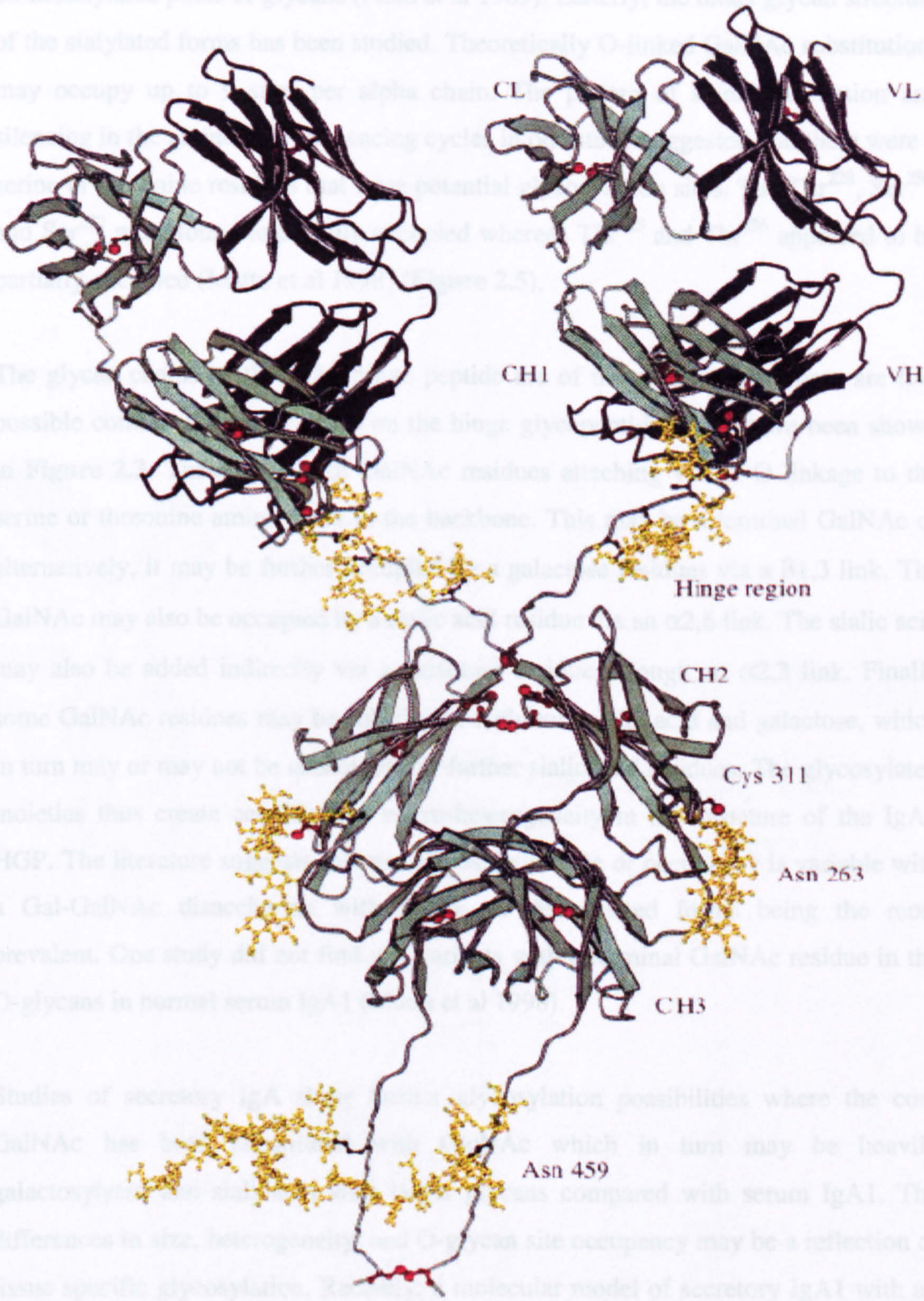



**Figure 2.4** Molecular model of human IgA1 showing the constant and variable light and heavy chains, the hinge region, tail piece and the N- and O-linked glycosylation sites with their glycan chains shown in yellow. Re-produced courtesy of Professor Raymond Dwek.

on desialylated pools of glycans (Field et al 1989). Latterly, the intact glycan structure of the sialylated forms has been studied. Theoretically O-linked GalNAc substitutions may occupy up to 9 sites per alpha chain. The pattern of signal depression and silencing in the amino acid sequencing cycles in one study suggested that there were 5 serine or threonine residues that were potential glycosylation sites. The $Thr^{228}$, $Ser^{230}$, and $Ser^{232}$ were found to be fully occupied whereas $Thr^{225}$ and $Thr^{236}$ appeared to be partially occupied (Mattu et al 1998) (**Figure 2.5**).

The glycan chains of the IgA1 hinge peptide are of the mucin type. There are five possible combinations of glycans on the hinge glycopeptide which have been shown in **Figure 2.3**. These consist of GalNAc residues attaching via an O linkage to the serine or threonine amino acids in the backbone. This may be a terminal GalNAc or alternatively, it may be further occupied by a galactose residues via a β1,3 link. The GalNAc may also be occupied by a sialic acid residue via an α2,6 link. The sialic acid may also be added indirectly via a galactose residue through an α2,3 link. Finally some GalNAc residues may be substituted with both sialic acid and galactose, which in turn may or may not be substituted by further sialic acid residues. The glycosylated moieties thus create considerable micro-heterogeneity in the structure of the IgA1 HGP. The literature suggests that the glycosylation site or occupancy is variable with a Gal-GalNAc disaccharide with mono- or desialylated forms being the most prevalent. One study did not find any variants with a terminal GalNAc residue in the O-glycans in normal serum IgA1 (Mattu et al 1998).

Studies of secretory IgA show further glycosylation possibilities where the core GalNAc has been substituted with GlcNAc which in turn may be heavily galactosylated and sialylated with larger glycans compared with serum IgA1. The differences in size, heterogeneity, and O-glycan site occupancy may be a reflection of tissue specific glycosylation. Recently, a molecular model of secretory IgA1 with all its glycans has been constructed, in which the Fab arms form a T shape and the SC is wrapped around the heavy chains (Royle et al 2003). The O-glycan regions on the heavy (H) chains and the SC N-glycans have been shown in this work to contain adhesin-binding glycan epitopes including galactose-linked β1,4 and β1,3 to GlcNAc, fucose-linked α1,3 and α1,4 to GlcNAc and α1,2 to galactose,

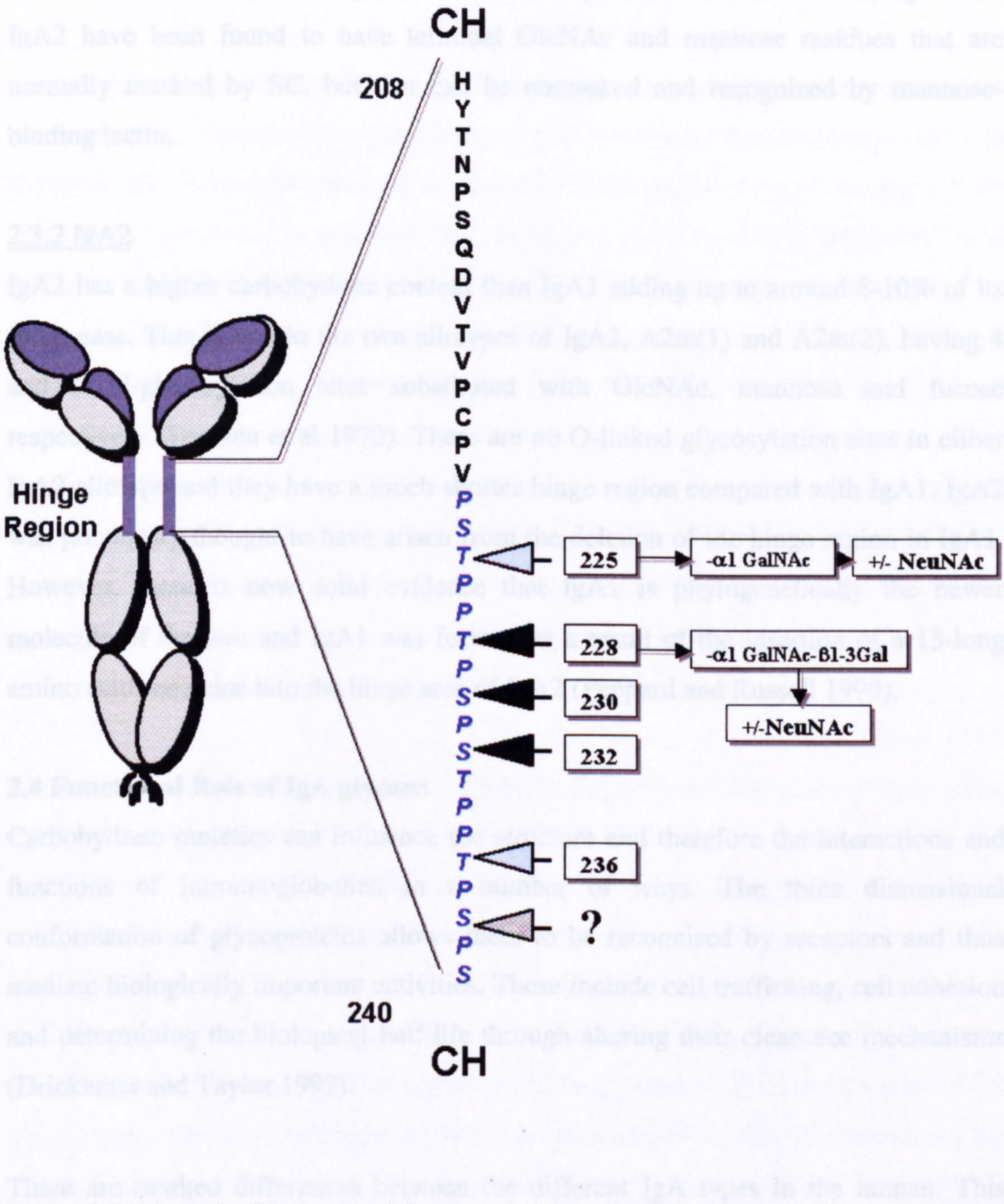



**Figure 2.5** Schematic diagramme of the serum IgA1 molecule indicating the hinge region and the 33 mer (208-240) hinge glycopeptide situated between the constant heavy chains 1 and 2 (CH1 and CH2) isolated after trypsin digestion. The amino acids in blue indicate the hinge glycopeptide core. The black arrows demarcate the fully occupied positions, and the grey arrows the partially occupied O-glycosylation sites in normal serum IgA1. Further glycans (?) may be located at any of the remaining serine (S) or threonine (T) residues. The various O-glycan permutations are indicated.

and α2,3 and α2,6-linked sialic acids. These epitopes are thought to provide secretory IgA with further bacterial binding sites. While the Fab region mediates adaptive immunity, these glycan epitopes will enable it to participate in innate immune reactions. Furthermore the N-glycans on the heavy chains of both secretory IgA1 and IgA2 have been found to have terminal GlcNAc and mannose residues that are normally masked by SC, but that can be unmasked and recognized by mannose-binding lectin.

### 2.3.2 IgA2

IgA2 has a higher carbohydrate content than IgA1 adding up to around 8-10% of its total mass. This is due to the two allotypes of IgA2, A2m(1) and A2m(2), having 4 and 5 N-glycosylation sites substituted with GlcNAc, mannose and fucose respectively (Tomana et al 1972). There are no O-linked glycosylation sites in either IgA2 allotype and they have a much shorter hinge region compared with IgA1. IgA2 was previously thought to have arisen from the deletion of the hinge region in IgA1. However, there is now solid evidence that IgA1 is phylogenetically the newer molecule of the two and IgA1 was formed as a result of the insertion of a 13-long amino acid sequence into the hinge area of IgA2 (Peppard and Russell 1999).

## 2.4 Functional Role of IgA glycans

Carbohydrate moieties can influence the structure and therefore the interactions and functions of immunoglobulins in a number of ways. The three dimensional conformation of glycoproteins allows them to be recognised by receptors and thus mediate biologically important activities. These include cell trafficking, cell adhesion and determining the biological half-life through altering their clearance mechanisms (Drickamer and Taylor 1993).

There are marked differences between the different IgA types in the human. This suggests that these differences may play a fundamental role in the biological functions of the IgA molecules. The glycans can be relatively bulky in size thus dictating the stability of the tertiary and quaternary structure of the molecule (Wright and Morrisson 1993). For example the presence of O-linked sugars on the repeating protein sequence will encourage a typical folding pattern conferring certain stability to the structure and permitting the creation of long mucin type domains. The chemical

properties of carbohydrate residues can also affect the whole of the structure. For example sialic acid is highly negatively charged and therefore quite labile and reactive. By enveloping the glycoprotein it can influence its interactions with other molecules and receptors (Lis and Sharon 1993).

Glycosylation also affects the assembly, transporting and surface expression and secretion of the glycoprotein. Carbohydrates also have many post-secretory effects on the molecule. They could prolong the half-life of the molecule by protecting it from proteolysis, increasing its solubility and decreasing immunogenicity (Montreuil et al 1982; Lis and Sharon 1993). IgA1 is normally very susceptible to degradation by bacterial proteases. This is not the case in IgA2 as the main focus of the attack is that of the hinge region which is absent in IgA2 (Kerr 1990). The heavier hinge region of secretory IgA1 may be more protected due to its bulk thus conferring an added conformational advantage to the molecule in the hostile mucosal environment (Wold et al 1994). One study has suggested that this appears to be particularly useful in the gastric mucosa where the extra glycosylation of the secretory IgA compared with serum IgA has been shown to inhibit the adherence of *Helicobacter pylori* to the gastric mucosal surface (Boren et al 1993).

The clearance of IgA may also be mediated by their glycans for example by binding to ASGP-R directly (Moldoveanu et al 1990). The hepatic ASGP-R binds the desialylated terminal Gal and GalNAc with a higher affinity for IgA1 rather than IgA2 where the O-linked sugars of its hinge region act as the ligand (Stockert et al 1982). One explanation for altered clearance of IgA has come from studying the properties of IgA circulating immune complexes (CIC) (Tomana et al 1999). They tend to be prone to self-aggregation and therefore significantly larger than normal. This could inhibit the normal catabolic pathways involved in their removal. The antibodies to the exposed glycoprotein core can also obscure the natural binding sites for the ASGP-R and prevent uptake and catabolism, leaving high levels of CIC for deposition in tissues such as the renal mesangium. Such large immune-complexes are also unable to cross the sinusoidal fenestrae in the space of Disse where the glycoproteins come into contact with the ASGP-R (Phillips et al 1986). The net electric charge of these CICs may also influence their distribution and deposition.

IgA is also cleared via the FcαR1 (CD89) on monocytes and other arms of the reticulo-endothelial system (Silvain et al 1995). The glycan epitopes on IgA can therefore theoretically have an effect on the Fc-mediated clearance of the molecule.

In addition, the ligand for the leukocyte Fc alpha-receptor lies within the CH2 domain of the IgA molecule in close proximity to the N-linked Asn 263 residues as well as the hinge glycopeptide region (Mazengera and Kerr 1990). The sugars are involved in the interaction between the ligand and its receptors as demonstrated by studies involving glycosylation site-directed mutagenesis and inhibition experiments with monosaccharides (Burton and Woof 1992, Keidan et al 1995). Human activated CD4 and CD8 T cells also express receptors that recognise O-linked sugars on the hinge regions of the IgA1 but not IgA2 (Rudd et al 1994).

IgA cannot fix complement by the classical pathway due to the lack of a C1q binding site. It can however activate the alternative pathway under certain circumstances (Kerr 1990). N-glycosylation of IgA has been shown to be pivotal in the binding of C3 and therefore activation of the alternative complement pathway (Zhang and Lachmann 1994). The authors used IgA2 in this study to avoid the confounding effects of the IgA1 O-glycans and therefore little is known about the role of IgA1 O-glycosylation in complement interactions.

## 2.5 Glycobiology of pathological conditions

### 2.5.1 Introduction

N- and O-oligosaccharide variants on glycoproteins (glycoforms) can lead to alterations in protein activity or function that may manifest themselves as overt disease. The number of diseases cited in the literature with abnormalities of protein glycosylation is an ever-increasing list. The proteins affected may be cell surface or free glycoproteins. These alterations range from physiological responses due to an acute condition to characteristic pathogenic aberration of the protein glycosylation pattern. In this section a summary of those diseases that are known to be the result of an inherited or acquired glycoprotein oligosaccharide structural alteration follows.

## 2.5.2 Congenital diseases

Alterations of O- and N-linked glycans in glycoproteins have been found in an ever-increasing number of congenital diseases. These conditions have been summarised in **Table 2.1.**

## 2.5.3 Acquired diseases

*2.5.3.1 Rheumatoid arthritis (RhA), juvenile RhA, SLE, Sjogren's:* Rheumatoid diseases are amongst the best know conditions where aberrant protein glycosylation exists. The abnormality occurs on the IgG molecule in particular the N-linked glycans at Asn 297 of the gamma heavy chain. Normally this moiety has the capacity to carry up to 3 galactose residues. Immune complexes from patients with rheumatoid arthritis are particularly rich in asialo-agalactosyl IgG (Bond et al 1996). The proportion of patients with under-glycosylated IgG is consistently higher than matched controls (Rademacher et al 1988). Similar changes have been found in SLE and Sjogren's syndrome (Axford and Hay 1991). The defect appears to be in the activity of the galactosyltransferase in both B and T cells, which is inversely proportional to the levels of the agalactosyl phenotype (Axford and Alavi 1995). This does not appear to be due to a gene locus abnormality, as B cell GTase activity can be stimulated with EBV (Axford and Alavi 1995). No inhibitors of the enzyme are known in these patients. The percentage of the abnormally glycosylated IgG correlates with poor prognosis and disease activity. The changes of IgG galactosylation with pregnancy (decreased levels of abnormal glycoforms are associated with remission and with increasing levels in post-partum relapse) also suggest a pathogenic role for abnormal glycosylation in RhA. Defectively galactosylated IgG are prone to self-aggregation and formation of complement fixing IgG complexes. They are also incapable of ligating B-cell Fc receptors causing disruption of the normal feedback suppression of B cells, which in turn leads to the expansion of auto-reactive B cell clones and auto-immunity.

*2.5.3.2 Tn polyagglutinability syndrome (TnPS):* In this rare haematological disease, red blood cells are lysed by normal ABO compatible serum. This is due to defective glycosylation of erythrocyte membrane proteins, glycophorins A and B, creating a neo-antigen to which individuals will have natural autoantibodies. These proteins are heavily glycosylated with an N-linked site and 14 O-linked residues. In TnPS, the

| Disease | Clinical Features | Biochemistry | References |
|---|---|---|---|
| Leroy Disease (lysosomal storage disease) | AR, mental retardation, death in 1st decade | Lack of Golgi GlcNAc phosphotransferase | Leroy et al 1970 |
| Congenital disorders of glycosylation (CDG) Ia/Ib and II | Ia/II: neurological dysfunction Ib: hepatic, intestinal disease | Ia/Ib: phosphomannomutase defect; II: GlcNAcase deficiency | Jaeken et al 1994 and 1997 |
| Leukocyte adhesion deficiency type II | Severe mental retardation | Absence of neutrophil sialyl Lewis x ? Secondary to fucose deficiency | Becker et al 1999 |
| HEMPAS | AR; anaemia, cirrhosis | Absence of poly N-acetyllactosamine | Fukuda 1999 |
| Wiskott-Aldrich Syndrome | X-linked, eczema, thrombocytopoenia, immunodeficiency | Lymphocyte O-linked glycan defect 2° to altered GlcNAc and sialyl trasferase activity | Higgins et al 1991 |
| Multiple forms of muscular dystrophy | Muscular dystrophy | Glycosylation defects of α dystroglycan | Martin et al 2003 |
| Walker-Warburg syndrome | Neuronal migration disorder causing brain abnormalities | Defective O-mannosylation | Martin-Rendon et al 2003 |
| Fukuyama congenital dystrophy | Muscular dystrophy | Genes mutations encoding glycosyltransferases | Martin-Rendon et al 2003 |

**Table 2.1** Congenital disorders associated with the presence of abnormal O- or N-linked glycosylation. AR: autosomal recessive, GlcNAc: N-acetylglucosamine, GlcNAcase: N-acetylglucosamine transferase, HEMPAS (Hereditary erythroblastic multinuclearity associated with positive acidified serum).

defect is either due to asialylation (T antigen named after Thomsen-Friedenrich) or due to agalactosylation (Tn antigen) the latter being due to loss of β1,3 galactosyltransferase activity (Cartron et al 1978). This in turn is because of gene repression rather than mutation (Thurnher et al 1993). **Figure 2.6** shows the structures of the different antigens found in different conditions.

*2.5.3.3 Other conditions associated with altered glycosylation*: Other conditions in which altered glycosylation have been observed include: inflammatory bowel disease, in particular Crohn's disease; infectious conditions such as periodontal disease, TB and leprosy; systemic vasculitides such as ANCA positive vasculitis (Holland et al 2002); and paroxysmal nocturnal haemoglobinuria. Furthermore, in conditions such as cystic fibrosis (CF), altered glycosylation of the secretory component of mucosal IgA has been found with evidence of undersialylation and overfucosylation (Marshall et al 2004). In addition, the CF glycophenotype is expressed on membrane glycoconjugates of CF airway epithelial cells as increased fucosyl residues in α1,3 and α1,4 linkage to N-acetyl glucosamine, decreased fucosyl residues in α1,2 linkage to galactose and decreased sialic acid. One study has found evidence supporting the hypothesis that wild type CF transmembrane conductance regulator acts in the Golgi. Its mutation in CF causes changes in the compartmentalization of terminal glycosyltransferases, thus creating the CF glycophenotype (Stoykova et al 2003).

Finally, increasingly the role of altered glycosylation of tumour antigens has become the subject of research. Many tumours are known to express a high level of tumour associated carbohydrate antigens which are associated with a more aggressive course and poor patient survival. Examples of this are the presence of Tn and Sialyl-Tn in colorectal, lung, and breast cancer (Hakomori 2001). Other glycoforms suppress tumour metastases, yet the biochemical mechanisms behind this are mostly unknown. In the case of breast cancer, a recent study suggested that a particular type of O-glycosylation was responsible for the inertness of tumor-associated MUC1 glycoforms expressed on the breast cancer cells to effective dendritic cell processing. This was mediated by the O-glycans masking the cleavage site on the peptide which is normally subject to proteolysis by antigen presenting cells (Hanisch et al 2003).

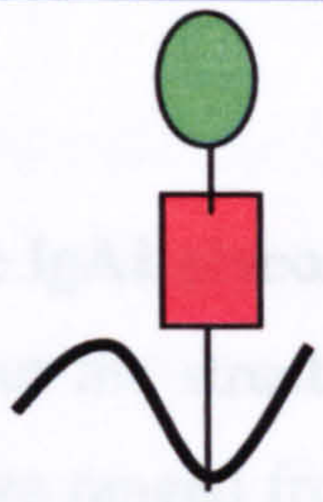
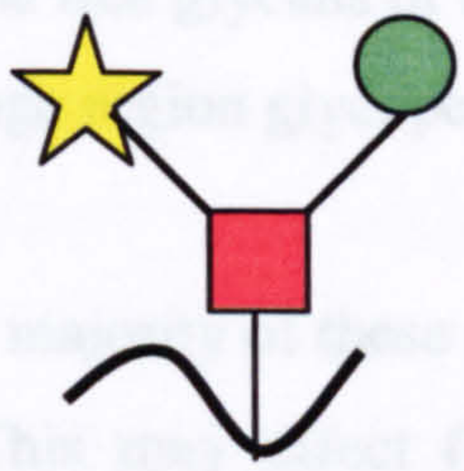
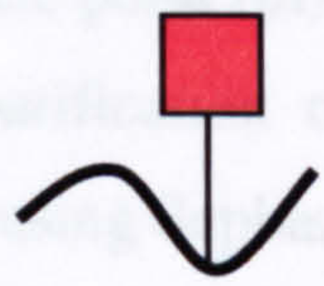
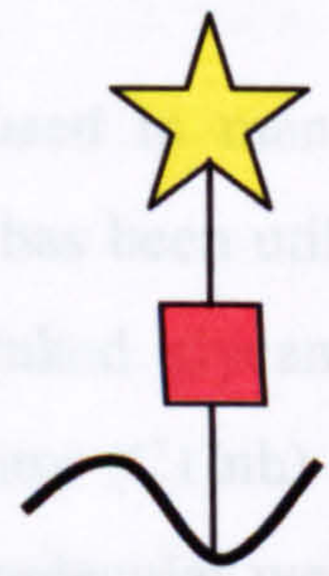
| | |
|---|---|
| **T**<br>**(Asialylated GalactosylGalNAc)** |  <br>**Ser/Thr** |
| **Sialyl-T**<br>**(Sialylated GalactosylGalNAc)** |  <br>**Ser/Thr** |
| **Tn**<br>**(AgalactosylGalNAc)** |  <br>**Ser/Thr** |
| **Sialyl-Tn**<br>**(Sialylated agalactosylGalNAc)** |  <br>**Ser/Thr** |

**Figure 2.6** Scheme of different antigens arising from the variable glycosylation of the serine/threonine (Ser/Thr) residues in O-linked glycopeptides. The red square represents the GalNAc residue which is O-linked to the protein back bone (shown in a black curved line). The green circle and yellow star represent galactose and sialic residues respectively.

## 2.6 Glycosylation of IgA1 in IgAN

### 2.6.1 Investigative tools in IgAN

A number of tools have been used in the study of the IgA1 glycosylation. Whilst each of these has added to the body of information about the structure of the molecule, each of them has their unique limitations. These have ranged from studies where the whole IgA1 molecule has been analysed using ELISA-type lectin-binding studies, to various chromatographic techniques of assessing the free glycans of the hinge peptide, and finally a mass spectrometric analysis of the hinge region glycopeptide.

It is important to note at this stage though, that the majority of these studies have used different methods to isolate and purify IgA1. This may affect the results of the glycosylation studies per se. Techniques such as gel filtration and ion exchange chromatography separate molecules by size alone. Given that many other proteins of similar size appear in the serum and that the isoelectric point (pI) of serum IgA is also variable, these two methods are unsuitable for purification of IgA1. Out of the immunological affinity techniques, chromatography using Sepharose columns damage the O-glycans in particular the labile sialic acids and ELISA-type techniques immobilise the IgA onto plates and therefore elution is not possible.

Jacalin affinity chromatography (JAC) has been used in many studies where the specific affinity of jacalin for the Galβ1,3-GalNAc has been utilised to isolate IgA1. As this binding is not specific to IgA, other O-linked glycans in the serum may contaminate the IgA1 preparation. C1esterase inhibitor (C1Inh) is one such molecule which may be separated out by precipitating high molecular weight (HMW) proteins and discarding the low molecular weight (LMW) components containing C1Inh. The other common contaminant may be IgG from its immune complexes with IgA1. However the main criticism of this method of IgA1 purification may be that the very O-glycans analysed are those used for purification and therefore there is a risk of losing any potential non-jacalin binding IgA1 glycoforms. Having said this, in studies where JAC and IgA1 immobilisation on immunoplates have been performed in parallel, good correlation has been found between the results (Allen 1999). From a practical point of view, this method is the only effective approach to purifying IgA1 and therefore until some other technique is made available, it is the best procedure at our disposal.

## 2.6.2 Lectins

Lectins are plant and animal derived proteins with specific affinity for carbohydrate ligands. The existence of erythrocyte-agglutinating proteins has been recognised since the turn of the 19th century. By the 1960s it had also become apparent that such proteins also agglutinate other types of cell in a sugar-specific manner. Although shown to occur widely in plants, and to some extent also in invertebrates, very few lectins had been isolated till the early 1970s. This changed with the recognition of lectins as extremely useful tools for the investigation of carbohydrates on cell surfaces, in particular of the changes that the latter undergo in malignancy, as well as their use in the isolation and characterization of glycoproteins. In recent years numerous lectins have been identified from plants, microorganisms, and animals, and the structures of hundreds of them has been elucidated. Concurrently, it has been shown, that lectins function as recognition molecules in cell-molecule and cell-cell interactions in a variety of biological systems (Sharon and Lis 2004).

Lectins are polyvalent proteins with multiple sugar binding sites and their binding can be inhibited by the appropriate oligosaccharide. In nature their function is variable but they are in general involved in recognition and binding processes. In scientific work, lectins are used as tools to identify glycan moieties and lectin binding is a simple method of screening for glycosylation changes in glycoproteins. They can be used in an ELISA-type set up. A variety of lectins with variable specificities are available which recognise different O-glycan moieties. **Table 2.2** summarises the most commonly used lectins for analysis of O-glycans in IgAN. Some of the lectins used in the assessment of IgA1 O-glycans are discussed below.

As will be discussed later, a number of lectins are available that have strong and specific affinity for O-linked glycans, the most commonly commercially available one being jacalin bound to agarose. Jacalin recognises the Gal β1,3 GalNAc link. Its binding is not affected by sialylation and it also has some affinity for agalactosylated moieties (Roque-Barreira and Campos-Neto 1985). Therefore it is assumed that it is capable of binding all glycoforms of IgA1. The most commonly used lectins in IgA1

| Lectin | Binding | Specificity |
|---|---|---|
| Artocarpus integrifolia (Jacalin) | Galβ1,3GalNAc and GalNAc | Not affected by NeuNAc |
| Amaranthus caudatus (AC) | Galβ1,3GalNAc | Partially inhibited by NeuNAc |
| Arachis hypogaea (Peanut agglutinin, PNA) | Strong affinity for asialylated Galβ1,3GalNAc | Highly inhibited by NeuNAc (low binding to native IgA1) |
| Vicia villosa (VV) | GalNAc | Inhibited by Gal/NeuNAc |
| Helix aspersa (HAA) | Affinity for GalNAc | Inhibited by Gal/partially inhibited by NeuNAc |
| Helix pomatia (HPA) | GalNAc | Inhibited by Gal/NeuNAc |
| Caragana arborescens (CAA) | GalNAc | Inhibited by Gal/NeuNAc |
| Bauhinia pupurea (BPA) | Galβ1,3GalNAc | Inhibited by NeuNAc |

**Table 2.2** Summary of a number of lectins used in the assessment of O-glycan carbohydrate structure. This table shows the preferential glycans the lectins bind to as well as the specificity for the oligosaccharides by which they are inhibited.

glycobiology are Vicia villosa (VV), Helix aspersa (HAA), and peanut agglutinin (PNA). VV and HAA both preferentially bind to O-glycans with GalNAc alone (T antigen) and are used as an indirect measure of the presence or lack of galactose (Osawa and Tsuji 1987). PNA however binds preferentially to the Gal-β1,3-GalNAc and is highly inhibited by the presence of sialic acids (Osawa and Tsuji 1987). Studying samples of IgA1 using a variety of lectins such as the examples cited above give an indirect picture of the glycan content of the glycopeptide.

2.6.3 Lectin binding studies

*2.6.3.1 Findings of lectin binding studies in IgAN* Although many studies have reported aberrant glycosylation of IgA1 in IgAN, the precise structural characteristics of this molecule have not been elucidated. The main strategies adopted, their strengths and shortcomings are discussed below. Andre et al first noted aberrations in IgA1 glycosylation in IgAN in 1990. He observed that IgA from patients with IgAN had reduced reactivity with the lectin jacalin which is highly specific for the β-1,3 galactose linked to GalNAc on the O-linked glycan chain. Although other groups independently confirmed these findings, others showed the reverse, where evidence of altered glycosylation was found as increased binding to jacalin (Tomino et al 1995; Hiki et al 1996). Other groups subsequently reported increased binding of IgA1 from sera of these patients to other lectins that recognise terminal GalNAc (Allen et al 1995; Baharaki et al 1996; Tomana et al 1997). These studies all suggested alterations in patients with IgAN using indirect evidence for degree of lectin binding of the intact or desialylated IgA1. Whilst some explained their findings as evidence of under-galactosylation, others interpreted their results as over or undersialylation or a combination of both.

*2.6.3.2 Limitations of lectin binding studies in IgAN* The main problem with these studies has been the use of a speculative and indirect technique which is subject to variation in the result depending on the source and specificity of the lectin used (Kobayashi et al 1988). Even when the same lectin has been used, as in the initial Andre paper (1990) as well as studies by Hiki et al (1996), and Tomana et al (1997 and 1999), conflicting results have been found about the differences in IgA1 jacalin binding. In one study that examined the interactions between IgA1 and different lectins with seemingly similar reactivity to glycan moieties of IgA1, contradictory

results were obtained (Allen et al 1995). One study even drew the wrong conclusion of increased sialylation from its finding of increased binding of IgA1 to PNA which on the contrary is known to be inhibited by the presence of sialic acid on the Gal β1,3-GalNAc (Baharaki et al 1996).

The other technical issue that may result in different findings has been the use of a variety of techniques in the isolation of IgA as well as the assessment of the binding. Whilst some groups have used anti-IgA antibodies to immobilise and purify the IgA1 molecule, others have used JAC which in itself may introduce a bias in terms of the characteristics of the purified IgA. Jacalin is supposed to be highly specific for the Gal β1,3-GalNAc residue and is not thought to be inhibited by the presence or absence of either galactose or sialic acid residues (Roque-Barreira and Campos-Neto 1985, Gregory et al 1987). However, there is no assurance from the literature that severely altered glycans are still bound by jacalin and it may be that the most abnormal and potentially pathological glycoforms are lost as a result of the JAC purification process employed by some of these studies.

Finally the other main limitation of these studies is that they were conducted on diverse groups of patients from vastly different racial backgrounds ranging from the original French series, to the Japanese, and American groups. The differences highlighted by the different groups could simply be a reflection of the genetic differences between the groups of patients and controls under study.

### 2.6.4 Reverse phase HPLC and gas liquid chromatography

More indirect evidence of abnormal glycosylation has come came from studies using various biochemical techniques such as gas-liquid chromatography and HPLC following chemical release of intact IgA1 O-glycans in their native form by hydrazinolysis (Patel et al 1993). Analysis of free O-glycans can be brought about using a number of techniques. Again, none of these can give precise information about O-glycan site occupancy but they do impart some knowledge of the structure and relative frequencies of the sugar residues. IgA O-glycans may be released chemically or enzymatically. The former is achieved by hydrazinolysis at 60 degrees Celsius to guarantee the release of intact O-glycans as opposed to any N-glycans. Enzymatic release of sialylated glycoforms is achieved only after treatment with

neuraminidase to remove sialic acid residues. Asialylated glycoforms may be released by O-glycanase or GalNAcases.

The total carbohydrate content of IgA1 O-glycans has been assessed in this way (Mestecky et al 1993). This study showed a decrease in the total galactose content found in serum IgA1 from patients with IgAN compared with normal controls. Hiki et al (1996) later demonstrated decreased ratio of higher to lower galactosylated forms of the IgA1 HGP in IgAN using rpHPLC to analyse the O-glycans released from IgA1 by hydrazinolysis. They showed a significant increase in the percentage of species containing asialylGalβ1,3GalNAc with a parallel decrease in the mono-sialyl species in IgAN. None of these studies however were able to produce a detailed report of the glycoforms or their stoichiometry. The studies using lectin binding and chromatographic techniques in analysing the IgA1 O-glycosylation have been summarised in **Table 2.3**.

2.6.5 Fluorophore assisted carbohydrate electrophoresis (FACE)

FACE is performed by labelling free O-glycans with a fluorophore which are then run on a polyacrylamide gel. The densities of the bands obtained under UV light correspond to the relative quantities of glycans present in the sample (Klock and Starr 1998). A paper by Allen et al (1999) using this method following the enzymatic and chemical release of IgA1 O-glycans has shown increased frequency of single GalNAc units in IgAN. This also corresponded well to binding with the GalNAc specific lectin Vicia Villosa.

2.6.6 Mass Spectrometry:

Mass spectrometry was successfully adopted for glycoprotein structure over a quarter of a century ago (Morris et al 1978). These early studies led to the discovery of multiple oligosaccharides O-linked through amino acid residues to the protein backbone. The methodology at the time involved the production of volatile derivatives using permethylation, which survives until the present day for some analyses. Glycopeptide analysis however is now best done using one of the more advanced soft ionisation techniques such as matrix assisted laser desorption ionisation

| METHOD | IgAN FINDING | COMMENT | REFERENCES |
|---|---|---|---|
| Jacalin binding | ↓ Binding to jacalin; First evidence of reduced galactose content | Calculated binding using relative index of the anti-IgA Ab dose-response curve against IgA. | Andre et al 1990 |
| Jacalin coated ELISA microplates with IgA1 from n=22 IgANs, n=14 renal and n=20 normal controls | ↑ Binding to jacalin suggesting ↑ Gal content of IgA1 from patients with IgAN | ? Due to different jacalin specificity or genetic differences | Tomino et al 1995 |
| ELISA based lectin binding, jacalin affinity chromatography purification N=20 IgAN N=20 Normal Controls | 1. ↑ VV/HA binding suggesting presence of increased terminal GalNAc in IgAN 2. No change in AC/PNA binding specific for fully galactosylated Galβ1,3 GalNAc | AC/PNA findings not consistent with VV/HA binding | Allen et al 1995 |
| 1. IF with Jacalin/ IgA1 and IgA2 mAb in renal tissue 2. Serum IgA1 (D-galactose inhibition assay) in 58 IgAN patients, 41 renal and 52 healthy controls | 1. Same reactivity in serum and mesangial IgA to anti-IgA1 and FITC-jacalin 2. ↑ Binding in 33% of IgAN patients vs. normals (3.8%) and other GNs (9.8%) | 1. Different jacalin source/specificity? 2. Racial differences? 3. Assay used: D-galactose inhibition assay limiting findings to IgA1 reactive to jacalin | Hiki et al 1996 |
| Sandwich ELISA lectin binding in 47 patients and controls | ↑Sialylation/↓ galactosylation | No renal controls, desialylated IgA1 | Baharaki et al 1996 |
| JAC, Gas-phase hydrazinolysis, HPLC | Undersialylation of IgA1 | Effect of hydrazinolysis on analysis | Hiki et al 1996 |
| 1. Carbohydrate content analysis: Gas-liquid chromatography 2. ELISA based lectin binding 3. Lectin binding both on intact and de-sialylated IgA | 1. ↑HAA/CAA binding of intact IgA1: 10 patients, 10 controls; ↑BPA/HPA binding of desialylated IgA1 from 10 IgAN vs. 10 normal and 9 renal controls | 1.↓ Galactose content in IgAN 2. Based on neuraminidase treated IgA1 3. Anti-IgA1 Ab purification avoids IgA1 loss | Tomana et al 1997 |
| Eluted glomerular IgA1 from nephrectomies, Lectin binding | ↑Lectin binding to VV, HAA of eluted IgA1 compared with serum IgA1 | Indirect evidence of altered deposited IgA1 glycosylation; Only 3 samples | Allen et al 2001 |

**Table 2.3** A summary of the investigations of IgA1 O-linked glycans in IgAN using a variety of techniques such as lectin-binding, chromatography, and inhibition assays.

(MALDI) (Dell and Morris 2001). Such studies are coupled with gas chromatography and other biochemical methods such as trypsin and exoglycosidase digestion to inform us of the detailed stoichiometry of glycoproteins.

*2.6.6.1 MALDI-MS* This is the most sensitive of the ionisation technologies. The sample is normally embedded in a LMW UV-absorbing matrix. A pulsed laser beam is then fired at the sample to effect ionisation. The matrix absorbs the laser energy and transfers the pulse to the sample in such a way that produces a singly charged molecular ion. This process does not produce very many fragments and it is therefore useful for screening molecular ions with high sensitivity (Dell and Morris 2001).

*2.6.6.2 Studies of IgA1 glycosylation using mass spectrometry* **Table 2.4** summarises the findings of different studies using mass spectrometry for the analysis of the glycan structure of IgA1. Only one group of investigators has successfully employed this technique for the analysis of O-glycans. As their technique has become more accurate, they have shown consistently that a proportion of serum IgA1 glycoforms from patients with IgAN show some degree of under galactosylation or under sialylation of the hinge peptide (Hiki et al 1998, Iwase et al 1998, Odani et al 2000, Hiki et al 2001). One paper has shown evidence of increased presence of TF antigen with small amounts of Tn and sialyl Tn antigens in IgAN (Iwase et al 2002). More importantly, Hiki et al in their paper published in 2001 also showed that the deposited IgA1 eluted from biopsy specimens from the kidneys of patients with IgAN, showed reduced levels of galactose and sialic acid. One recent paper has also shown that cultured B-cells from tonsils of patients with IgAN, when cultured, produce undersialylated and undergalactosylated IgA1 (Horie et al 2003). In short, over the last 8 years, tremendous effort has been put into analysing the structure of the IgA1 hinge region and its O-glycans and so far mass spectrometry has produced the best results. The spectra obtained however are not clear enough to give a definitive view of the structure, stoichiometry and the spatial relationship of the glycans with the protein backbone and with each other. At best this technique has offered comparisons of ratios of different glycoforms with each other and a definitive quantitative and qualitative comparison of the glycosylation of IgA1 hinge structure from normal and diseased serum is still out of reach.

| METHOD | FINDINGS IN IGAN | COMMENT | REFERENCE |
|---|---|---|---|
| MALDI ToF MS Sequential de-glycosylation | No clear peaks obtained from intact IgA1 but multiple glycoforms were identified post de-sialylation | Evidence for multiple glycoforms in normal IgA1 | Iwase et al 1996 |
| JAC, MALDI N=13 IgAN N=11 other GN N=8 normal controls | Suggestive of defect in Gal and/or GalNAc residues | Only Galβ1,3GalNAc analysed due to poor spectra | Hiki et al 1998 |
| MALDI, endo-peptidase treatment | Determined further glycoforms of IgA1 HGP | Study of normal serum IgA1 | Iwase et al 1998 |
| Electrospray Ionised liquid MS | Decreased GalNAc, Gal and NeuNAc | Pooled sera used IgAN n=4, renal controls n=10, normals n=5 | Odani et al 2000 |
| Eluted glomerular IgA1 from 291 biopsy samples serum IgA1 n=2 for normal controls and IgAN, MALDI | Decreased sialylation and galactosylation | First direct information about deposited IgA1 | Hiki et al 2001 |
| IgA glycosidase treatment to assess sugar side chain ratios | Increased numbers of TF antigens | | Iwase et al 2002 |
| MALDI on IgA1 from cultured tonsillar B-cells | Increased percentage of asialo/agalacto-type of IgA1 in 57% of IgAN patients ($p<0.04$) | IgAN n=7 Chronic tonsillitis n=5 | Horie et al 2003 |

**Table 2.4** A summary of IgA1 O-glycan analyses using mass spectrometry and the results obtained in IgAN and normal serum. These data have suggested the presence of under-galactosylated and undersialylated species both in the serum and in mesangial deposits of patients with IgAN but have failed to produce spectra that yield detailed data on the glycoform structure.

*2.6.6.3 Problems with MS in the analysis of IgA1* Whilst mass spectrometry has been successful in the quantitative analysis of relative frequencies of N- and O- glycoforms of glycopeptides, there have been a number of difficulties encountered in studying the glycosylation pattern of IgA1 hinge peptide. This part of the IgA1 molecule is a short sequence of amino acids with a high density of O-glycans. It would therefore make MALDI an attractive method for identifying the O-glycan structure. Unfortunately, the glycopeptides obtained from digested IgA1 in their native glycosylation state contain a wide array of glycoforms. This means the spectra obtained show wide, poorly differentiated peaks. This has been the main stumbling block for definitive analyses of IgA1 glycosylation using this technique. Hiki et al (2001) have partially overcome this problem by enzymatically removing sialic acid residues from the molecule, as these sugars are bulky and notoriously difficult for analysing on MALDI mass spectrometers. This has allowed some insight into the number of configuration of the core sugars of the hinge peptide but not that of the intact molecule.

*2.6.6.4 Summary of MS findings* At present the consensus opinion on the role of glycosylation in the pathogenesis of IgAN is that a fraction of the IgA1 molecules in the blood of these patients is under glycosylated, exposing the underlying hinge region to immune-complex formation with naturally occurring antibodies. These immune-complexes may then interact with components within the renal mesangium where they are deposited and set off an inflammatory reaction. IgA circulating immune complexes have been found to contain under-galactosylated IgA1 (Tomana et al 1997). A recent paper by Allen et al showed IgA1 eluted from nephrectomy specimens of 3 IgAN patients had reduced jacalin binding and increased binding to lectins specific for GalNAc lending indirect support to the notion that deposited IgA1 in the renal mesangium is under glycosylated (Allen et al 2001). Hiki Y et al 2001 also analysed IgA1 extracted from pooled renal biopsy samples and found mass spectroscopic evidence of under-galactosylation in deposited IgA1. The bulk of evidence lends partial support to the hypothesis that serum and deposited IgA1 is under-glycosylated in IgAN although many of the findings have been contradictory or confounded by technical difficulties.

2.6.7 Summary:

The first suggestion that IgA glycosylation may be abnormal and contribute to the pathogenesis of IgAN was made nearly 15 years ago (Andre et al 1990). Since then there has been much research interest and progress towards the identification the structure and role of these glycans in IgAN. Despite the attraction of the theory and some convincing experimental data, no definite role for the aberrant glycosylation of IgA1 in the pathogenesis of IgAN has been found. This is predominantly due to the uncertainty regarding what normal glycosylation pattern in IgA1 is as well as the technical difficulties in assessing protein glycosylation listed above. In the search for IgA1 glycosylation abnormalities, the results of different studies, based on a whole host of techniques has produced widely varied results. These contradictory findings may on the one hand be a reflection of the racially and genetically diverse populations under study, or they simply reflect the absence of a unified approach to the elucidation of the structure of these glycans. There is much more to be learnt about the exact nature of the IgA1 hinge region and its glycans of both normal and diseased serum and their interactions with the receptors and effector cells, which may have a pivotal role in the pathogenesis of IgAN. This highlights a clear need for a definitive technique to unravel the glycosylation pattern of the IgA1 molecule in normal serum and in those with diseases such as IgAN.

# Chapter 3: Methods and Materials

## 3.1 Patient recruitment

### 3.1.1 Subjects

Patients and normal subjects were recruited according to the guidelines of the local Ethics Committee. No patients were recruited under 18 years and over 75 years of age. Other exclusions included the diagnosis of HIV and diabetes as well as patients of Afro-Caribbean extraction. The majority of patients were recruited from the King's College Hospital renal and liver units and controls were from members of staff at the hospital and the laboratories at King's College and Guy's Hospitals. Dr Alice Smith of Leicester University donated a number of sera from patients with known primary IgAN, myeloma, myeloma and Henoch-Schönlein Purpura (HSP), and normal controls.

*Patient groups:*

A. Patients with histological diagnosis of primary IgAN

B. Patients with histological diagnosis of hepatic IgAN

C. Patients with HSP secondary to IgA myeloma

*Control groups:*

D. Patients with histological diagnosis of Non-IgA associated renal disease (e.g. membranous nephropathy, mesangioproliferative GN, minimal change nephropathy, and focal and segmental glomerulosclerosis (FSGS)) as controls for group A

E. Subjects with hepatic cirrhosis with either biopsy proven or clinical absence of evidence of glomerular disease serving as controls for group B

F. Patients with IgA myeloma with no renal involvement as controls for group C

G. Normal healthy controls for both groups A and B and C

Patients were matched for age but not gender. Subjects under the care of the liver unit with no previous histological diagnosis were screened for evidence of renal involvement. Those with evidence of nephritis, i.e. presence of haematuria, greater than 1+ proteinuria, and/or creatinine above the normal range were referred to a renal clinic and enrolled in the appropriate group according to diagnosis. All subjects were recruited with full informed consent. Normal controls and patients with hepatic cirrhosis with no evidence of renal disease were not biopsied. Normal renal function

was inferred from the presence of a normal serum creatinine, blood pressure, and urinalysis.

### 3.1.2 Samples

Urine and blood samples were obtained to identify potential patients for recruitment for the study as well as samples for the investigations. Venous blood was obtained from all subjects for laboratory tests of renal function and immunological assessment, namely serum creatinine, urea, and immunoglobulin levels. Blood samples taken for experimental purposes were collected in heparinised tubes. Peripheral blood mononuclear cell and monocyte preparations were separated from blood by density gradient centrifugation as described below. Serum was separated by centrifuging a clotted sample at 2383Xg for 10 minutes. The serum was then frozen immediately in 1ml aliquots at minus 20°C for future analysis. Urine samples were examined by dipstik urinalysis. Renal biopsy samples were used to classify subjects according to histological diagnoses.

## 3.2 Serum Separation

25ml of venous blood were collected into heparinised tubes and placed on ice to maintain at 4°C. The samples were centrifuged at 941Xg for 10 minutes at 4°C in a refrigerated RT-6000 centrifuge. The plasma was removed and a pinch of protamine sulphate (Sigma P2162) and 2 drops of thrombin (Sigma, T9549) were added. This was then left to clot for 2 hours after which the tubes were spun at 2383Xg and the supernatant serum was frozen in 1ml aliquots at -20°C.

## 3.3 PBMC Culture

### 3.3.1 Materials and Solutions

Lymphoprep Solution (Robbins Scientific Catalogue No. 221397)

50 ml Leucosep tubes (Greiner No 227290)

96 Microwell flat bottom culture plates (Nagle Nunc Int., Rochester, USA 160377)

RPMI plus glutamine culture medium 1640 (Gibco BRL, Paisely, UK)

$RPMI^{+}$

Foetal Calf Serum (Sigma, Poole, Dorset, UK, F 3371)

Pokeweed mitogen (PWM) (Sigma L9379)

Trypan Blue (Sigma T6146)

Hepes Buffer 1 molar (Sigma H0887)

Sodium pyruvate solution 100mM (Sigma S8636)

L-Glutamine/Penicillin/Streptomycin 200mM (Sigma G6784)

### 3.3.2 Procedure

The cell preparation was performed under sterile conditions in a good primary cell culture laboratory. All the work was carried out at 4°C on ice or in the refrigerated RT-6000 centrifuge. After removing the serum, cells from the collected whole blood were re-suspended in RPMI up to their original volume and mixed gently. PBMCs were then separated by density-gradient centrifugation which isolates cells based on their size and density (Graziani-Bowering GM et al 1997). A Leucosep tube was filled with 16mls of Lymphoprep solution and spun at 280Xg for 1 minute until the fluid settled below the Leucosep disc. 15mls of the reconstituted cells in RPMI were layered onto each Leucosep tube and centrifuged at 941Xg for 20 minutes at 4°C with brakes off. The PBMC layer was aspirated and washed twice with 25 ml of RPMI and then centrifuged at 280Xg for 10 minutes each time with brakes on. The pellet of cells was then re-suspended in 1ml of RPMI$^+$ and counted in a Neubar haemocytometer by taking 20µl of the cell preparation and adding 180µl of Tryphan Blue to make a 1:10 dilution.

The cells were then suspended at 1 million per ml in RPMI$^+$ in 96-well flat-bottom plates to be cultured for 7 days. To prepare the RPMI$^+$ solution, 65ml of Gibco RPMI with glutamine was removed from a 500ml bottle containing the solution. 50ml (10%) of thawed Sigma F-3371 Foetal Calf Serum was then added to RPMI. A further 5ml (1%) of Hepes buffer, Pyruvate and defrosted mixture of Penicillin/Streptomycin/Glutamine solutions each was added to the RPMI/FCS mixture. This was mixed well and filtered. The wells were filled with 100 microlitres of RPMI$^+$ or PWM/ RPMI$^+$ and 100 microlitres of cells were added to each to make up the appropriate dilution per well, i.e. $10^6$ cells per millilitre or $10^5$ cells per well. Blank cells were used as negative controls in two central columns. Stimulated cultures had PWM in RPMI$^+$ at 0.125µg/ml concentration. The supernatant was removed and

stored at –20 degrees Celsius for later analysis after seven-day incubation at 37° C and with 5% $CO_2$.

**3.4 Leukocyte CD89 Expression by flow cytometry:**

3.4.1 Materials and solutions

*Antibodies (Ab):*

-Fc block or anti-CD16/32 Ab (Pharmingen Cat. No. 01241A) for preventing non-specific binding

-α-CD89 Ab Phycoerythrin (PE) conjugated (Becton Dickinson, Oxford, UK Cat No. 33535X) for binding to Fcα receptor on leucocytes

-α-CD14 Ab (FITC-conjugated) (Dako, Ely, UK Cat No. F084401) for identifying monocyte population

-FITC-conjugated IgG1κ (Dako X092701) an irrelevant mouse antibody used as a negative control

-α-CD45 Ab RPE-conjugated (Dako R708701) as positive control as universally present on all leucocytes

*Solutions*:

-Phosphate buffer albumin prepared from 500ml phosphate buffered saline (PBS) (5 Phosphate Buffered Saline (Sigma P5368) tablets added to 500 mls de-ionised water) to which 0.5g Bovine Serum Albumin (Sigma F3371) plus 0.25 g Sodium Azide (Sigma S2002) were added.

-FACS&LYSE (Becton Dickinson Cat. No. 349202) solution: 20mls of a 1:10 dilution in distilled water.

*Cell line*:

U937 immortalised monocyte cell line donated by Dr Alice Allen of Leicester University. The original cells had been obtained from ATCC (CRL 1593).

3.4.2 Methods

5 ml of whole venous blood was collected into an EDTA vacutainer and maintained at 4°C. A 50:50 solution of PBA (0.5 ml) and whole blood (0.5 ml) was prepared and

mixed well. 5µl of Fc block (α CD16/32 Pharmingen Cat. No. 01241A) was added to the tube to make stock solution. This was to avoid non-specific binding by saturating all non-specific binding sites.

Flow (Falcon) tubes were labeled prior to addition of 100µl of the stock solution as follows: Fc block only, α-CD89, α-CD14, mouse IgG1κ, and α-CD45. The contents of each tube were stained with their respective antibodies at 5µl per tube. These were agitated and incubated at 4 degrees Celsius for 20 minutes. 2mls of FACS & LYSE solution was added to each tube and left at room temperature for 10 minutes to lyse the red blood cells. The tubes were then centrifuged at 941Xg for 5 minutes and the supernatant was discarded. The cells were washed once more in PBA and finally re-suspend in 1ml of PBA.

U937 cells were used as positive controls. They were cultured in Cellstar 75cm$^2$ tissue culture flasks with membrane filter packs and incubated in a fully humidified air atmosphere containing 5% $CO_2$ at 37°C, maintained in standard culture medium. $10^7$ U937 cells were used for each experiment after a minimum of three passages. They were treated as above with the exception of the FACS & LYSE step, where they were mixed with PBA solution instead. 100 µl of cells was placed into 2 separate tubes. 10µl of Fc block was added to both tubes as well as 5µl of α CD89 antibody to one, using the second sample as an unstained control.

All tubes were then read on the Coulter Epics Flow Cytometer on the following settings. The U937 readings were taken from their respective protocol, where the settings were gated on the cell line. Acquisition was set to read up to 20,000 events. For the leucocytes the device was set for counting a minimum of 2000 monocytes. The protocol was set to gate monocytes, neutrophils and lymphocytes. The measure of median luminescence was used for analyses.

### 3.5 Serum IgA1 Purification using Jacalin-Agarose Chromatography:

3.5.1 Materials and solutions

-Ammonium sulphate solution: 28g of ammonium sulphate (Sigma A4915) in 50mls of PBS (1 tablet in 50ml Ultra High Quality Water (UHQW))

-TRIS HCl 0.175M pH 7.5: 21.2g of TRIS base (Sigma T6791) was dissolved in 1litre of UHQW and stirred with a magnetic stirrer. When dissolved pH was adjusted to 7.5 with HCl. Stored at 4 degrees.

-0.1M melibiose (Sigma M5500): 4.1g in 120ml of TRIS HCl

-0.8M galactose (Sigma G0625) in TRIS HCl: 8.64g in 60ml of TRIS HCl

-Jacalin Storage Solution: 2.383g of HEPES in 1 litre of UHQW pH 7.5 (10mM); plus 8.766g of NaCl (0.15M); 0.0147g CaCl (0.1mM); 3.61g galactose (20mM); 7.22g lactose (20mM); 0.8mg Na Azide (0.08%).

-Jacalin Agarose (Vector Laboratories L1153; Batch L0229 used throughout all experiments)

-Snakeskin pleated dialysis tubing (Pierce Cat No 68100)

3.5.2 Method

IgA1 was purified based on a modified jacalin affinity chromatography method (Iwase H et al 1996). This technique is highly sensitive for recognising Galβ1,3-GalNAc and therefore appropriate for purifying IgA1 from serum. Frozen serum containing the equivalent of 4mg of IgA was thawed at room temperature. Equal volumes (50:50) of saturated ammonium sulphate solution and serum was pipetted into Starstedt tubes. The mixture was agitated for 20 minutes before centrifuging at 20780Xg for 10 minutes in the microfuge. The supernatant containing low molecular weight proteins was discarded and the pellet containing high molecular weight proteins was reconstituted in TRIS HCl up to the original volume of serum. (The LMW proteins contain molecules such as C1 esterase inhibitors, which also contain O-linked glycans, and therefore would confound results of glycosylation results of the study). The HMW protein pellet was dissolved using the vortex and then agitated for 30 minutes.

12 jacalin agarose columns were prepared by pipetting 1ml of well-mixed jacalin into a filter column (3ml spe filtration column). These were placed on a vacuum manifold on which a buffer exchange was performed by washing the column with at least 5 column volumes of TRIS HCl. The sample containing the IgA-TRIS solution was added to each column and allowed to drain to waste. This was then washed through with 10 column volumes of TRIS HCl and 5mls of 0.8M glucose in TRIS to release non-specifically bound protein, before eluting with 10mls of 0.1M melibiose/TRIS

HCl. (This volume for elution was selected based on an experiment where 0.5ml fractions of eluted IgA1 was collected in separate tubes and the protein content assessed on a UV 280 spectrophotometer and found to be maximal in the first 6mls of flow through).

The flow through containing the eluted IgA1 was collected in separate Snakeskin pleated dialysis tubing molecular weight cut-off of 10,000 Dalton. The columns were regenerated after each use by washing with TRIS HCl and preserving in jacalin storage solution at 4° C. The solution containing the IgA1 was dialysed at 4° C in distilled water for 2 days, changing the water three times a day. The sample was lyophilised overnight and reconstituted in 100 microlitres of distilled water and stored at -20°C.

An ELISA performed on purified IgA1 confirmed the presence of IgA1 and showed that this method produced a yield of 60%.

### 3.6 Isolation of IgA1 from breast milk

Breast milk was expressed into sterile containers at various times during lactation period. It was stored at -20°C immediately and saved for future use. IgA separation was performed using a method described by Kerr et al 1997. After de-frosting, 8ml of milk was mixed with an equal volume of isotonic saline. The solution was centrifuged at 10,000g for 1 hour at 4°C. The clarified milk was separated from the pellet of cell debris and the surface layer of fat. The pH was then lowered with HCl to 4 to precipitate out the casein. This was then centrifuged again at 30,000g for 30 minutes at 4°C. The supernatant was removed and neutralised to pH 7.0 with 2M TRIS HCl solution after which it was re-centrifuged as before and the supernatant was used to purify IgA1 by jacalin affinity chromatography as described above in section 3.5.

### 3.7 Isolation of IgA1 hinge glycopeptide

#### 3.7.1 Materials and solutions

-8M Urea buffer: 0.35M TRIS; 4mM EDTA pH 8.6: 24g urea dissolved in 25ml water on heater stirrer plus 2.2g TRIS and 0.075g di-sodium EDTA. pH was adjusted with HCl and made up to 50ml when cooled. Stored at 4°C.

-DTT/urea solution: 2 mg of dithiothreitol (Sigma D5545) per ml of urea solution

-2M formic Acid: 250µl of formic acid (Sigma F0507) in 3 ml of UHQW

-0.5M AMBIC: 0.8g $NH_4HCO_3$ in 20ml UHQW

-Methanol 'AnalaR'

-5% Acetic Acid (glacial) 'AnalaR'

-50/50 solution of methanol and 5% acetic acid

-Trypsin (Sigma T-1426)

-Strata C18 columns (6ml)

### 3.7.2 Reduction/alkylation

The reduction alkylation steps were based on a modification of the method by Iwase H et al 1996. Purified IgA1 was subjected sequentially to disulphide-reduction by dithiothreitol, alkylation with 4-vinylpyridine and trypsin digestion. Purified IgA1 was dissolved in 1ml of ultra high quality water (UHQW). A 30µl aliquot was removed and frozen at -20°C for future measurement of ratio of polymeric versus monomeric IgA1 by size exclusion chromatography. A further 100µl was saved for IgA1 ELISA. 50ml of 8M urea/0.35M TRIS/4mM EDTA buffer pH 8.6 was made and 500µl of it was added per 1mg of IgA1. The protein was reduced by adding 5.4µl of DTT/Urea solution per 1 mg of IgA1 in glass containers and incubated at 37°C for 4 hours. After cooling to room temperature, 6.4µl of 4-vinyl pyridine was added to each sample for recarboxymethylation and incubated for 90 minutes at room temperature. The reaction was stopped with 200µl of 2M formic acid per sample. The reduced and pyridylethylated IgA1 was dialysed against UHQW in a 10kDa Snakeskin dialysis membrane for 72 hours at 4°C and lyophilised in preparation for trypsin digestion.

### 3.7.3 Trypsin digestion

This was carried out by dissolving samples in 600µl of 0.5M ammonium bicarbonate to which 40µg of trypsin and 0.05% Na Azide were added (i.e. 100:1 IgA to trypsin ratio). After an overnight incubation at 37°C a further 20µg (50:1) of trypsin was added. The tryptic digest was then lyophilised to remove the AMBIC.

### 3.7.4 Hinge Glycopeptide isolation

The lyophilised tryptic digest samples were dissolved in 2ml 0.175M TRIS buffer pH7.5. The hinge glycopeptide was eluted with 10ml 0.1M Melibiose in TRIS HCl on a jacalin column. The samples were desalted on 6ml Strata C18 columns (Phenomenex, Ca USA), which had been primed first with 6ml methanol and then with 12ml 5% acetic acid. After applying the samples to the columns and allowing the effluent to run to waste, the columns were washed with at least 3 column volumes of 5% acetic acid on a vacuum manifold. The desalted HGP was eluted into glass containers in 6ml of 50:50 methanol/5%acetic acid solution and lyophilised.

This entire process produced a pool of 33mer hinge glycopeptides with an unusual trypsin cleavage on the C-terminal side of serine 240 which has been attributed to the presence, in the reduced and alkylated protein, of two adjacent pyridylethylated cysteines (residues 241 and 242) with the amino acid sequence of the isolated tryptic fragment being (208)HYTNPSQDVTVPCPVPSTPPTPSPSTPPTPSPS(240) (residue numbers are those of intact IgA1).

### 3.7.5 Purification of HGP by rpHPLC

The presence of purified IgA HGP was confirmed by rpHPLC. The glycopeptides containing O-linked glycans were separated from all the remaining peptides by the same JAC method as before. The run-off from the column containing the miscellaneous peptides was discarded and the eluted O-linked HGP was collected as shown in **figure 3.1.**

The purified HGP was reconstituted in 200µl of UHQW and applied to a PLRP-S300 rpHPLC column (150x4.6mmx8µm). Elution was performed using a linear gradient for 25 minutes from 8-48% acetonitrile in 0.1% trifluoroacetic acid with a flow rate of 1ml/min. Detection was made by monitoring the UV absorption at 214 nanometers. Under these chromatographic conditions, the HGP peak was observed at approximately 9 minutes (**figure 3.1**). The material eluted at peak position was collected in glass containers and lyophilised.

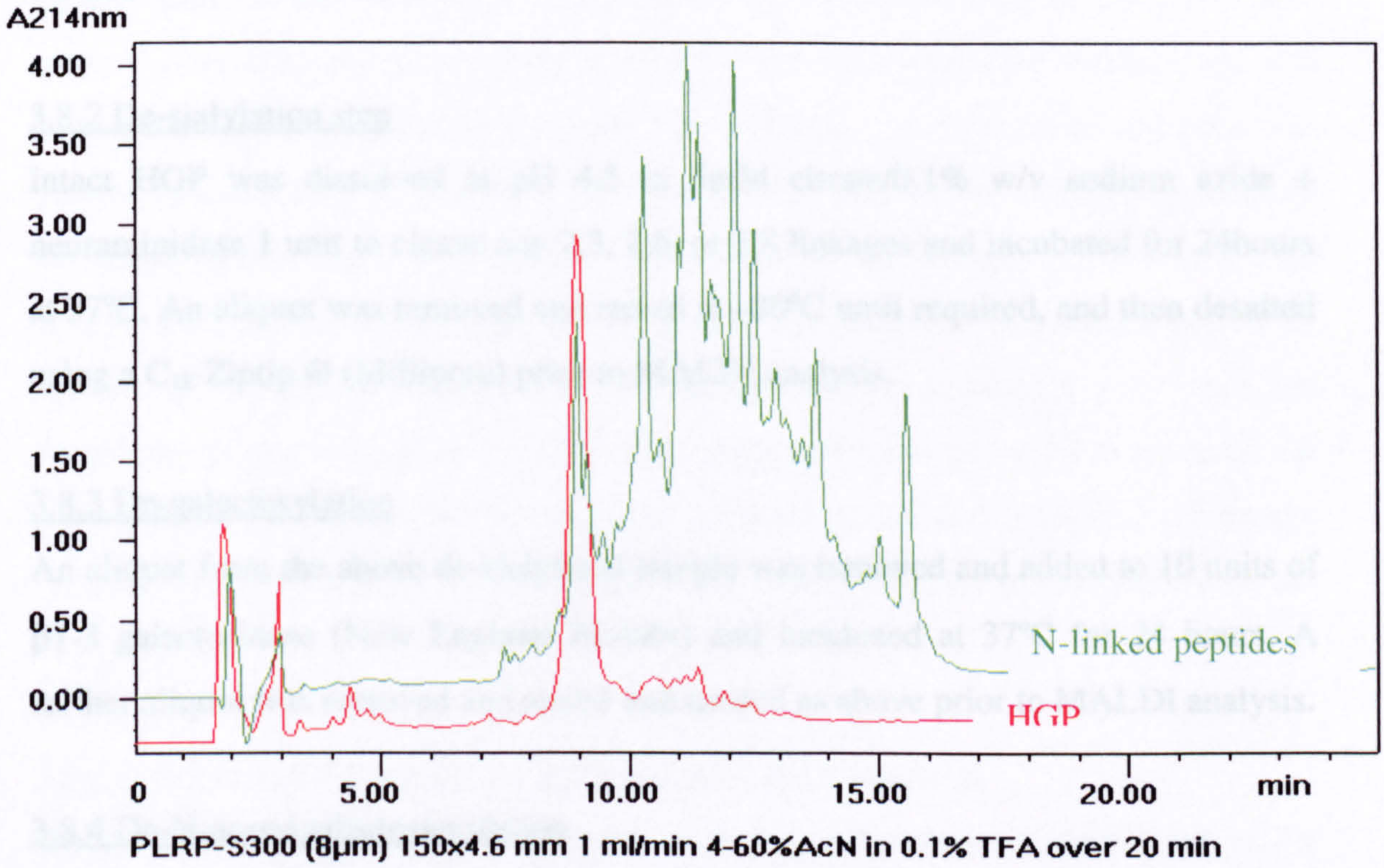



**Figure 3.1** Reverse phase HPLC trace of eluted IgA1 hinge glycopeptide (HGP) and tryptic digest in run-off. Green trace is the discarded flow through containing a mixture of N-peptides obtained from the tryptic digest of IgA1. The red trace contains the peak observed at 9 minutes, representing the O-glycosylated IgA1 HGP, which was collected for further processing.

## 3.8 Hinge glycopeptide de-glycosylation

### 3.8.1 Materials and solutions

-Neuraminidase (Sigma N2133) in 5mM citrate/0.1% w/v sodium azide

-β 1-3 galactosidase (New England Biolabs P0726S)

-α-N-acetyl galactosaminidase (NEB Cat. No. P0734S) in 5mM phosphate buffer pH 7.5 and 0.1% sodium azide

### 3.8.2 De-sialylation step

Intact HGP was dissolved at pH 4.5 in 5mM citrate/0.1% w/v sodium azide + neuraminidase 1 unit to cleave any 2,3, 2,6, or 2,8 linkages and incubated for 24hours at 37°C. An aliquot was removed and stored at –20°C until required, and then desalted using a $C_{18}$ Ziptip ® (Millipore) prior to MALDI analysis.

### 3.8.3 De-galactosylation

An aliquot from the above de-sialylated sample was removed and added to 10 units of β1-3 galactosidase (New England Biolabs) and incubated at 37°C for 24 hours. A further aliquot was removed and stored and treated as above prior to MALDI analysis.

### 3.8.4 De-N-acetylgalactosamylation

After de-galactosylation the sample was mixed in 5mM phosphate buffer pH 7.5, 0.1% sodium azide and 40 (NEB) units of α-N-acetyl galactosaminidase (NEB). This enzyme catalyses the hydrolysis of terminal α-GalNAc linkages in oligosaccharides. The solution was incubated again as above and analysed by mass spectrometry.

## 3.9 Matrix assisted laser desorption and ionisation time of flight mass spectrometric (MALDI-ToF-MS) analysis of IgA1 HGP

### 3.9.1 Materials and solutions

2mg/ml THAP matrix in acetonitrile and ammonium acetate solution: prepared by dissolving 2mg of 2,4,6-3 hydroxyacetophenon hydrate (Sigma T6460-2) in a solution of 250 µl acetonitrile and 750µl of 20mM ammonium acetate.

### 3.9.2 MALDI analyses

Lyophilised intact HGP samples were dissolved in 20µl of UHQW and a glycopeptide pool at a total concentration of 50-100pmol/µl (0.5µl) was applied to the MALDI target plates. Whilst still in liquid form, 0.5µl matrix was added and the mixture instantly vacuum dried in less than one minute. The first samples were analysed by matrix assisted linear desorption time of flight mass spectrometry on the Voyager DE-mass spectrometer (PerSeptive Biosystems) and later on a Kratos Axima CFR mass spectrometer (Kratos Analytical, Ltd, Manchester UK) operating in positive linear mode. Accelerated and grid voltage of 25,000V and 94% were used respectively. 200 shots were applied per analysed sample. The calibration of the mass/charge ratio was performed using bovine insulin (5737). The precise composition of the O-glycans in the hinge region of IgA1 was analysed by comparing the obtained peaks with a table of calculated masses of the hinge region peptide plus all possible combinations of O-glycans to identify the constituents of each peak. The percentage peak area per glycoform as a percentage of the whole spectrum peak area was used to compare the preponderance of one glycoform against the others within and between each group. Furthermore, the de-glycosylated samples were used for further verification of the HGP structure.

### 3.9.3 Java programme for handling and analysis of mass spectra

The data were handled in two different ways. Initially manual manipulation of the relative abundance of the glycoforms was attempted. This was a laborious task and prone to inaccuracies and so a Java programme was specifically devised by Mr Howard Smith of Leicester University for automating the handling of the spectral analyses. This programme automatically produces a list of the identified individual glycoforms from the m/z values fed into it. It also highlights m/z values that remain unassigned and matches them to a peak which may be a sodiated or potassiated form of a potential glycoform. Any further unidentifiable and unassigned peaks are then marked as U. For example the m/z of 4385 was one such peak that was encountered repeatedly in the spectra but the programme was unable to match it to any of the glycans listed in **Table 4.1** (presented in chapter 4), or to any other unusual oligosaccharide side chain permutations. The programme calculates the peak area for each individual glycoform as a percentage of the total area for each spectrum

produced. A mean percentage of peak area was then calculated for the patients and controls in each group. The programme also facilitates the ranking of the species in terms of their relative abundance. Finally, a value for the percentage sialylation and galactosylation of the GalNAc residues for each species is calculated.

## 3.10 Serum and supernatant Immunoglobulin detection

### 3.10.1 Materials and Solutions

*Antibodies*

-Rabbit anti-human IgA Ab (Dako A0262)

-Rabbit anti-human IgG Ab (Dako A0423)

-Rabbit anti-human IgM Ab (Dako A425)

-Mouse anti-human IgA monoclonal Ab (Oxoid M26012)

-Mouse anti-human IgA1 monoclonal Ab (Sigma I7262)

-Mouse anti-human IgA2 monoclonal Ab (Becton Dickinson 345110)

-Mouse anti-human IgG monoclonal Ab (Sigma I6260)

-Mouse anti-human IgM monoclonal Ab (Sigma I6385)

-Rabbit anti-mouse IgG alkaline phosphatase conjugate (Sigma A1902)

*Solutions*

-Tween 20 (Sigma P1379)

-WHO (World Health Organisation) standard reference serum

-p-nitrophenol phosphate substrate (1mg/ml) (Sigma N1891)

-Diethanolamine buffer pH 9.8 (Sigma D8885)

-3M Sodium hydroxide

### 3.10.2 Detection of total serum IgA and its sub-classes

A sandwich ELISA technique was used to measure total IgA, IgA1 and IgA2 concentrations. Microtitre plates were coated with 100ul of a 1:1000 dilution of rabbit anti-human IgA in PBS azide by incubation at 37 °C for 2 h. The plates were washed three times with PBS/Tween 20 solution and subsequent non-specific binding was blocked by incubation at 37 °C for 2h with 200ul of PBS containing 0.5% bovine serum albumin (BSA) and 0.05% Tween20 (T20).

Doubling dilutions of serum samples were made from 1:4000 to 1:128000 for IgA, 1: 1000 to 1:16000 for IgA1 and IgA2. Reference WHO serum with known concentration (2.4mg/ml) of IgA immunoglobulin was used as standard sera. Standard curves were constructed from 10 serial dilutions, in duplicate, beginning at 1:2000 and later adjusted to mg/ml. The plates were washed three times with PBS/T20 and serum IgA bound during overnight incubation at 4 °C was then detected by incubation at 2h at 37° C with 100ul of mouse monoclonal anti-IgA (diluted 1:1000), IgA1 (1:500), or IgA2 (1:500). The plates were washed thrice, after which 100ul of rabbit anti-mouse IgG alkaline phosphatase conjugate 1:1000 was added and plates incubated for 1h at 37° C. Phosphatase activity was assessed at room temperature with 100ul p-nitrophenol phosphate substrate (1mg/ml) dissolved in diethanolamine buffer (pH 9.8). The reaction were allowed to proceed unit sufficient yellow colour had developed (10 –15 min) and were then stopped by the addition of 50ul of 3M sodium hydroxide prior to reading of the plates in a Dynatech automated ELISA reader at 405nm.

The results were expressed as IgA, IgA1 or IgA2 mg/ml calculated from the standard curve obtained from the WHO reference serum expressing IgA antibody concentration as 2.4mg/ml. The value for each dilution of serum falling in the standard curve was taken and the value for the sample calculated as the mean of the six separate dilutions.

3.10.3 IgG detection in serum

Microtitre plates were coated with 100ul of a 1:520 dilution of rabbit anti-human IgG in PBS azide by incubation at 37 °C for 2 h. Serum sample dilutions were 1:8000 – 1:256000. Reference WHO serum with known concentration (12mg/ml) of IgG immunoglobulin was used as standard sera. Standard curves were constructed from 10 serial dilutions, in duplicate, beginning at 1:8000 and later adjusted to mg/ml. The remainder of the procedure was as for IgA detection.

3.10.4 IgA detection in supernatant

Microtitre plates were coated with 100ul of a 1:600 dilution of rabbit anti-human IgA (Dakopatts) in PBS azide by incubation at 37 °C for 2 h. Serum sample dilutions were 1:4–1:128. Reference WHO serum with known concentration of IgA immunoglobulin

was used as standard sera. Standard curves were constructed from 10 serial dilutions, in duplicate, beginning at 1:2000 and later adjusted to mg/ml. The remainder of the procedure was as for serum IgA detection.

### 3.10.5 IgG detection in supernatant

Microtitre plates were coated with 100ul of a 1:500 dilution of rabbit anti-human IgG in PBS azide by incubation at 37 °C for 2 h. Serum sample dilutions were 1:4–1:128. Reference WHO serum with known concentration of IgG was utilised. Immunoglobulin detection was used as per standard sera. Standard curves were constructed from 10 serial dilutions, in duplicate, beginning at 1:4000 and later adjusted to mg/ml. Rest of the procedure was per serum IgG detection.

### 3.10.6 IgM detection in supernatant

Microtitre plates were coated with 100ul of a 1:500 dilution of rabbit anti-human IgM in PBS azide by incubation at 37 °C for 2 h. Serum sample dilutions were 1:4–1:128. Reference WHO serum with known concentration of IgM was utilised. Immunoglobulin detection was used as per standard sera. Standard curves were constructed from 10 serial dilutions, in duplicate, beginning at 1:4000 and later adjusted to mg/ml. Rest of the procedure was as described above.

### 3.10.7 Statistical analyses

The results are expressed as mean ± SEM per group. An unpaired Student's t-test was used to compare the means for each group. All data analyses were undertaken using the Prism statistics package.

## 3.11 ELISA-type Lectin binding assays

These experiments were performed by Dr Alice Smith in the renal laboratories at the University of Leicester on samples collected by Dr Smith from patients at Leicester General Hospital: 13 with primary IgAN, 7 normal controls, 4 patients with IgA myeloma, and 2 with HSP secondary to IgA myeloma. Serum samples were collected and stored as above. The samples were tested using a method described by Allen et al (1995) against a panel of the following lectins: Vivia Villosa (VV), Peanut agglutinin (PNA), and Helix Aspersa (HAA). Briefly IgA1 was immobilised either indirectly

onto immunoplates using anti-IgA1 antibodies or directly by using purified IgA1 from jacalin affinity chromatography. A biotinylated lectin which binds to specific IgA1 O-glycans was then applied followed by the application of a peroxidase-conjugated avidin which binds the biotin in the previous layer. The colour reaction development was through the action of peroxidase on a chromogenic substrate. The absorbances detected by an automated plate reader were proportional to the amount of lectin bound. The results were adjusted for the IgA1 captured on the wells.

### 3.12 Measurements of polymeric to monomeric serum IgA ratio by size exclusion chromatography

Size exclusion chromatography was used to determine the different molecular forms of IgA in the sera of our patient and control groups. HPLC analyses were conducted using a Gilson HPLC equipped with 2 Gilson 306 pumps, one 302 pump, 1 model 118 UV detector at 214 nm, a Gilson model 715 PC-based control and data collection system, and a Gilson model 234 autosampler (Anachem, Luton, UK). The column used was a Tosoh TSK3000 SW column (Anachem, Luton, UK) with column dimensions of 30 cm long x 7.8mm in diameter. The column was calibrated using two mixes of calibrants containing proteins with molecular weights ranging from 66-669 kDa as per **figures 3.2** and **3.3**. 50µl of each sample was injected into the column at a flow rate of 0.5ml/min. The chromatograms were normalised using chromatograms of identical standards. The peaks obtained were then assigned a molecular form of IgA depending on the molecular weight, the integration having been based on the MW fractions derived from the standard proteins (**Figure 3.4**). The ratio of the polymeric to monomeric IgA was calculated and compared between the groups. Results were shown as +/-SEM and analysed by Mann-Whitney U test. P value of $<0.05$ were taken as statistically significant differences.

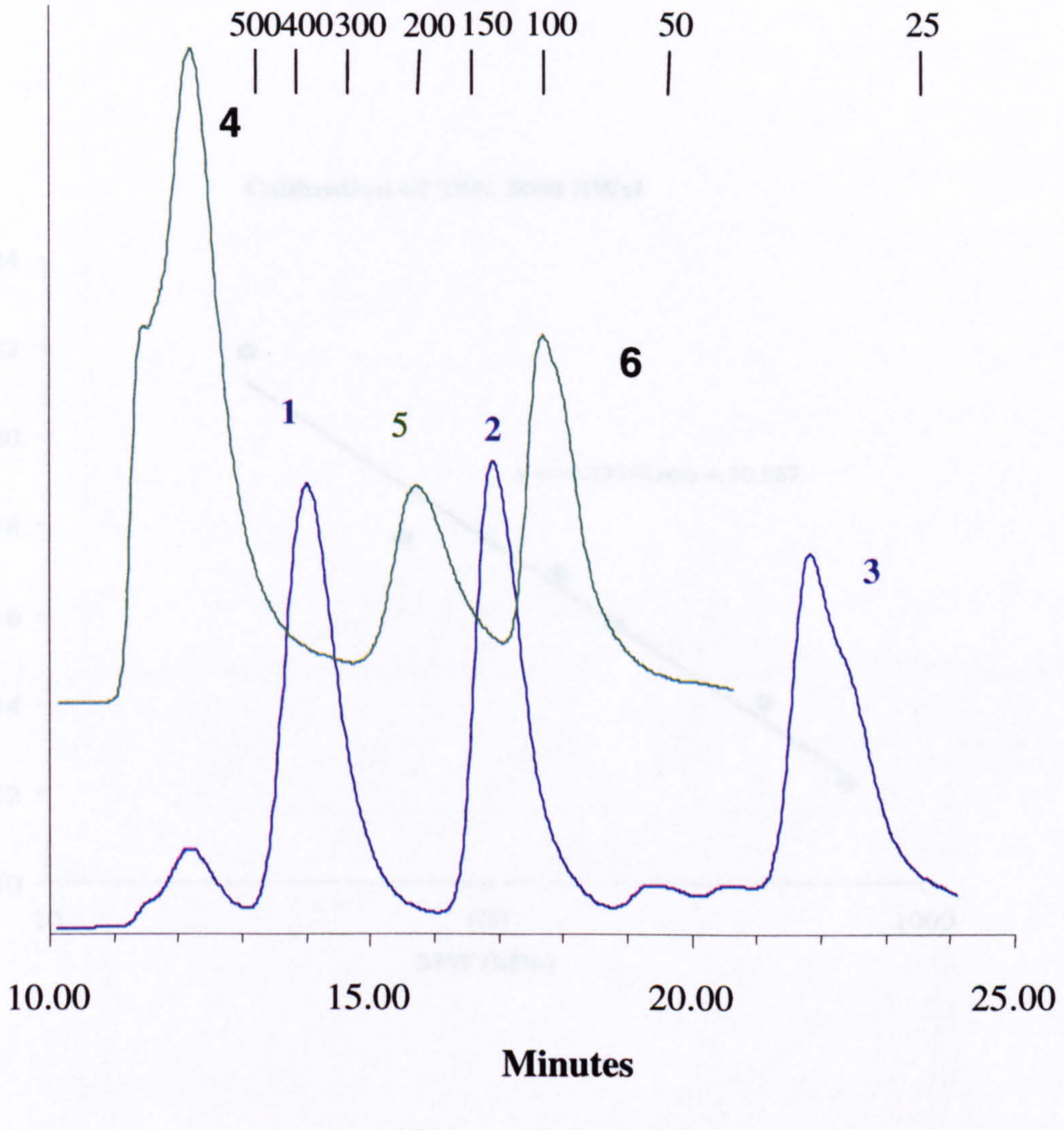



**Figure 3.2** The superimposed traces of proteins used to calibrate the size exclusion column for the analysis of the IgA polymers. Two mixes of calibrants were run. Mix A (blue trace) produced 3 peaks 1, 2, and 3 corresponding to Apo ferritin (MW 443 kDa), Alcohol dehydrogenase (MW 150kDa), and Carbonic Anhydrase (MW 29kDa) respectively. Mix B (green trace) came up as a further 3 peaks, with peak 4 representing thyroglobulin (MW 669 kDa), peak 5 was β-amylase (MW 200kDa), and peak 6 which was bovine serum albumin with a molecular weight of 66kDa.

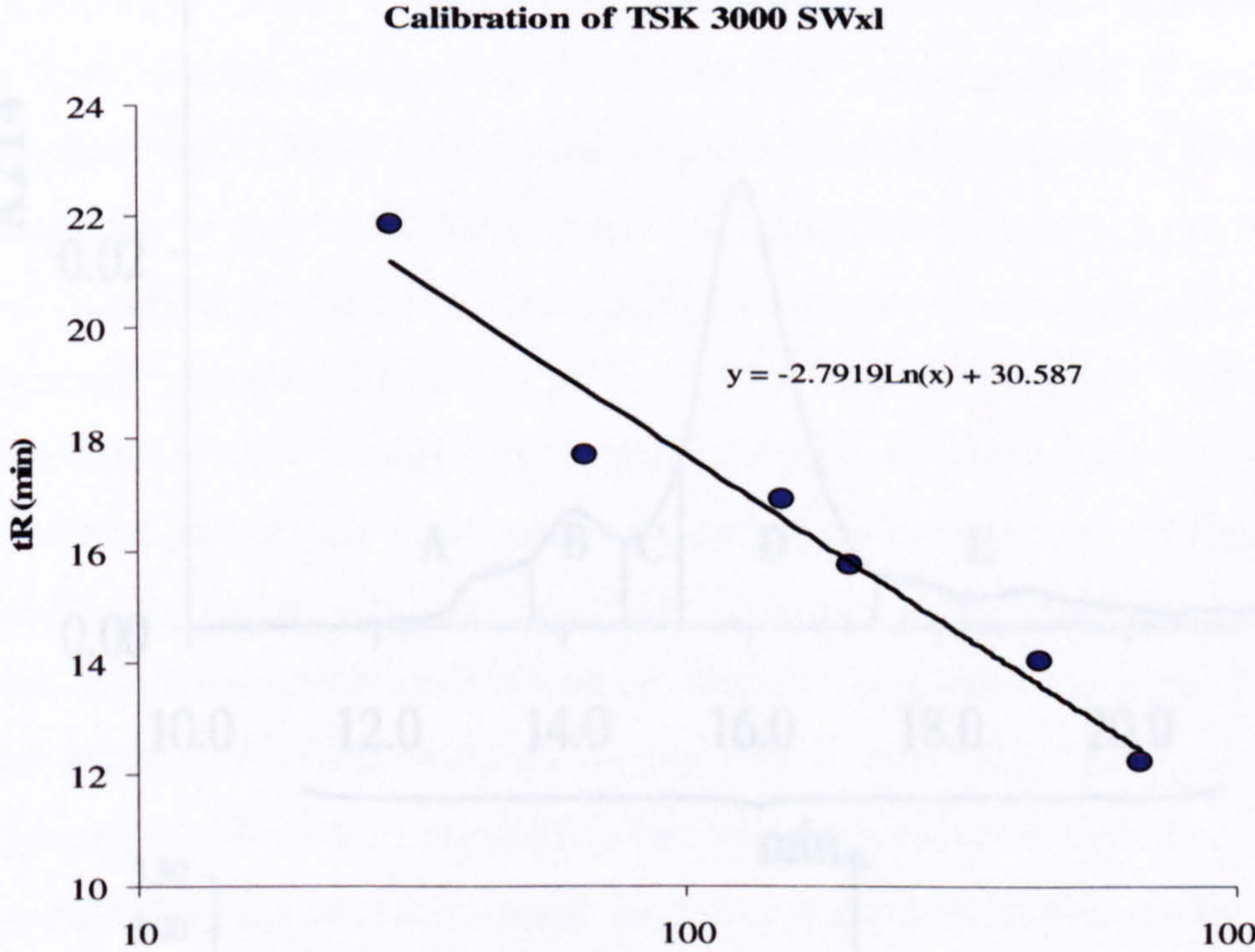



**Figure 3.3** The calibration of the TSK 3000 size exclusion column with a good correlation between retention time (tR) and the molecular weight (MW) of the calibrants in kDa.

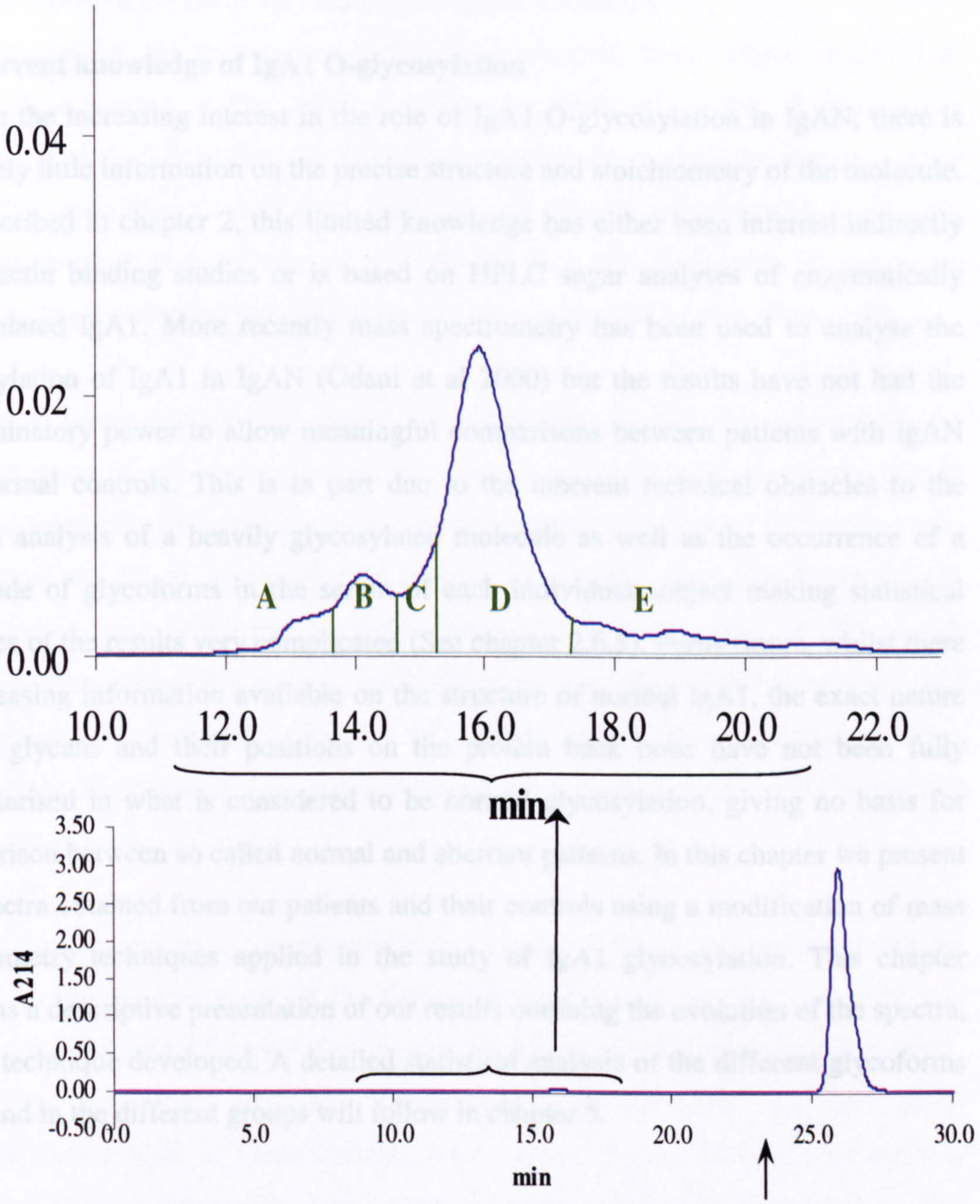



**Figure 3.4** Sample chromatograms showing a normalised peak with different molecular weight fractions coming off the size exclusion column at different times. The higher molecular weight species have the shorter retention times and vice versa. In this chromatogram the peaks have been normalised to the azide peak which was found in all the samples. Peak A contains species with a molecular weight of greater than 680 kDa, B has molecules with a molecular weight of between 450-680 kDa, C contains those between 350-450 kDa, D between 150-350 kDa, and E those less than 150 kDa.

# Chapter 4: IgA1 hinge glycopeptide O-glycosylation

## 4.1 Current knowledge of IgA1 O-glycosylation

Despite the increasing interest in the role of IgA1 O-glycosylation in IgAN, there is relatively little information on the precise structure and stoichiometry of the molecule. As described in chapter 2, this limited knowledge has either been inferred indirectly from lectin binding studies or is based on HPLC sugar analyses of enzymatically manipulated IgA1. More recently mass spectrometry has been used to analyse the glycosylation of IgA1 in IgAN (Odani et al 2000) but the results have not had the discriminatory power to allow meaningful comparisons between patients with IgAN and normal controls. This is in part due to the inherent technical obstacles to the precise analysis of a heavily glycosylated molecule as well as the occurrence of a multitude of glycoforms in the serum of each individual subject making statistical analyses of the results very complicated (See chapter 2.6.5). Furthermore, whilst there is increasing information available on the structure of normal IgA1, the exact nature of the glycans and their positions on the protein back bone have not been fully characterised in what is considered to be normal glycosylation, giving no basis for comparison between so called normal and aberrant patterns. In this chapter we present the spectra obtained from our patients and their controls using a modification of mass spectrometry techniques applied in the study of IgA1 glycosylation. This chapter contains a descriptive presentation of our results outlining the evolution of the spectra, as our technique developed. A detailed statistical analysis of the different glycoforms we found in the different groups will follow in chapter 5.

## 4.2 Identification of IgA1 by mass spectrometry

The entire tryptic digest of IgA1 was analysed by mass spectrometry using bovine serum albumin (BSA) as its control. The spectra obtained were of poor quality and a clear peak was not seen initially. A peptide mass fingerprinting tool (MS-Fit) from the University of California at San Francisco that produces a best fit list from matching mass spectrometry data to a protein sequence in an existing database was used. This database can suggest the identity of the user's protein. When used on the tryptic digest of IgA1, it matched the findings with a number of proteins including human Ig alpha-1 heavy chain constant region. It also correctly identified the BSA.

## 4.3 Initial identification of IgA hinge glycopeptide spectra

Mass spectra for the hinge glycopeptide were obtained from samples purified as described in chapter 3. The initial samples were analysed on the PE Biosystems Voyager System. These spectra which fell consistently within the expected wide 4200-6500 mass/charge range were identifiable as the hinge glycopeptide previously obtained by other studies (Iwase et al 1998). As with data from other studies in the literature, the baseline in many of the spectra was elevated and noisy (**Figure 4.1**).

In order to verify that this was indeed the hinge glycopeptide, the masses from the different peaks in any given spectrum were subtracted from each other and a pattern emerged where the differences in mass between peaks corresponded to the difference in the mass of a single oligosaccharide. For example in the spectrum in **figure 4.2**, the difference in mass between the peaks assigned 6017 and 5814 is 203 which corresponds to a mass difference of one GalNAc residue. Mass differences of 291 and 162 represent the presence or absence of a single sialic acid and galactose residue respectively. Furthermore the masses obtained were within 0.5-1% of the calculated glycopeptide mass.

Further experiments using de-glycosylating enzymes definitively confirmed that the spectra produced were those of the various glycoforms of the IgA1 hinge region. **Figures 4.3, 4.4,** and **4.5** present the progressive loss of glycans from a single sample, which has undergone sequential de-sialylation, de-galactosylation, and de-N-acetylgalactosamylation. These spectra show that the glycopeptide is progressively simplified through loss of its outer oligosaccharide coat until the denuded peptide backbone is left representing a single discrete peak at 3478 (calculated mass 3478), which corresponds to the previously calculated mass of the 33mer amino acid chain obtained by the reduction/pyridylation/tryptic digestion of IgA1 (Hiki et al 1997). **Table 4.1** shows the calculated values for each potential glycoform of IgA1 HGP.

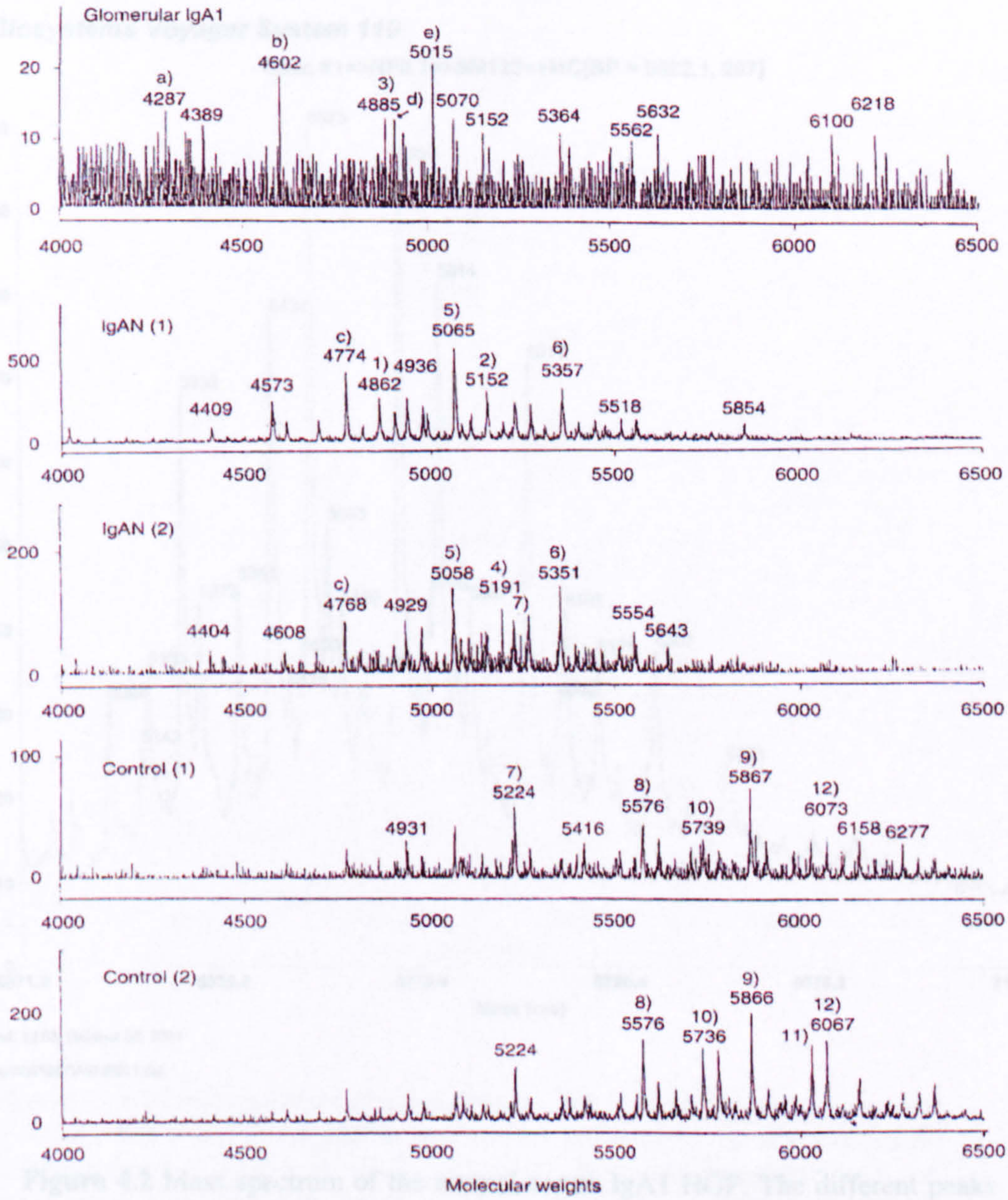



**Fig. 3. Mass spectra of IgA1 hinge in five samples.** The distribution of the peaks of glomerular IgA1 and IgA1 of the IgAN groups (upper 3 graphs) shifts to a molecular weight lower than in control groups (lower 2 graphs).

**Figure 4.1** An example of the quality of mass spectra obtained by other investigators of IgA1 hinge O-glycosylation using MALDI MS. Figures reproduced courtesy of Dr Y Hiki.

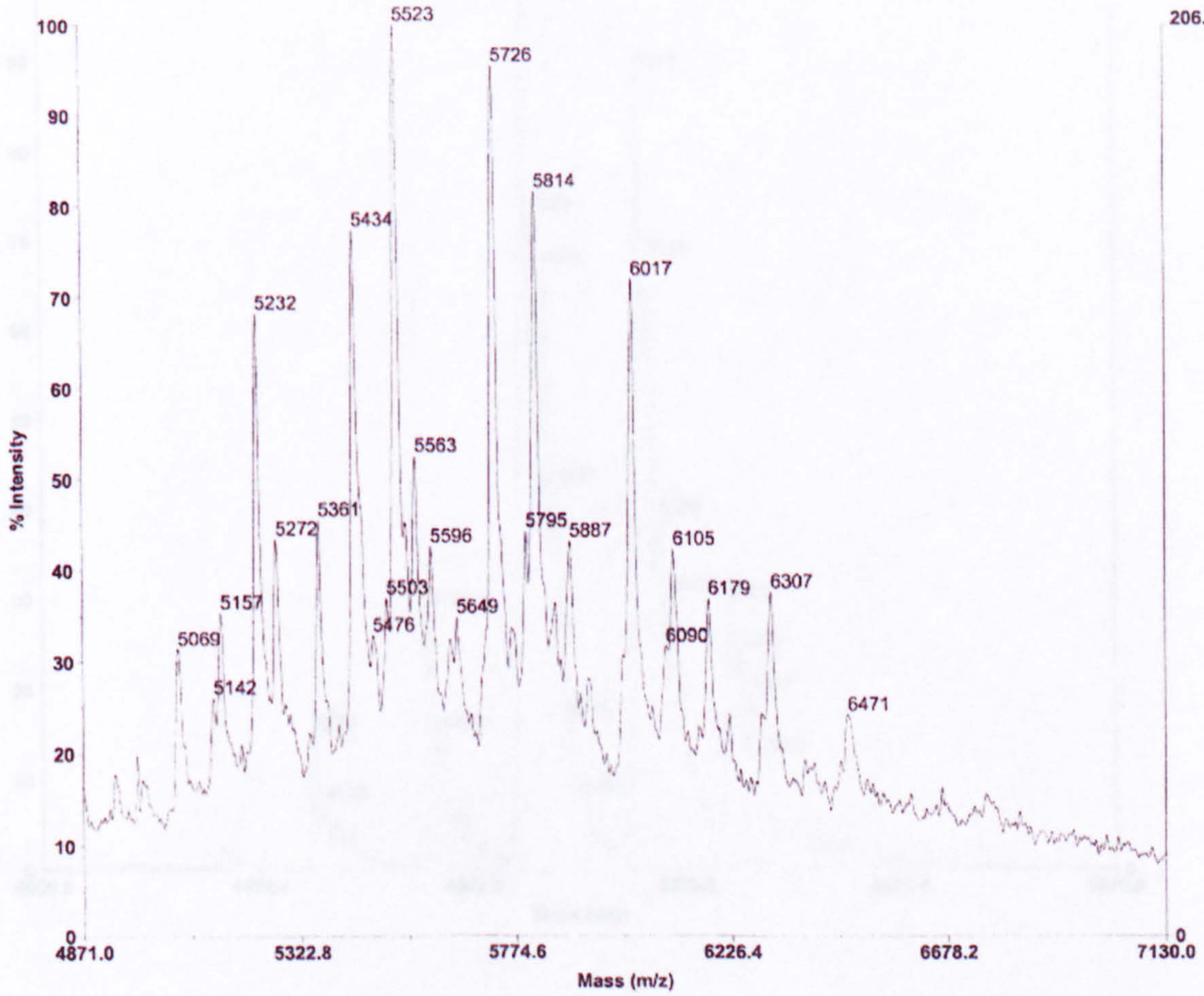



**Figure 4.2** Mass spectrum of the normal serum IgA1 HGP. The different peaks represent the glycoforms with variable degrees of glycosylation. For example the observed peak at 6471 (calculated mass 6468) represents a glycoform containing 5 GalNAc, 5 Gal, and 4 NeuNAc residues. The next observed peak (6307) is a glycoform comprising 5GalNAc, 4 Gal, and 4 NeuNAc residues with the m/z difference of 161 being due to the extra Gal on the former glycoform.

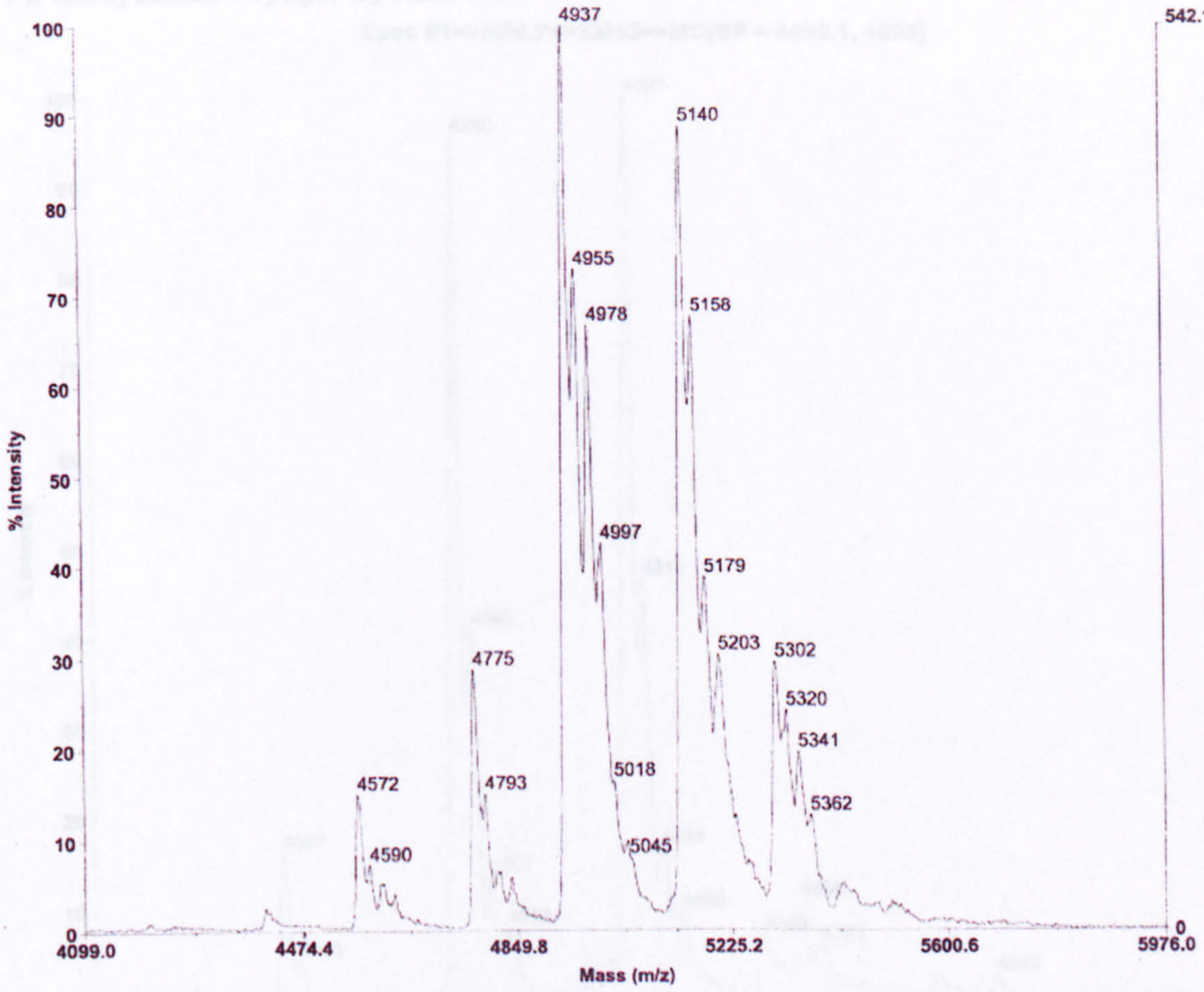



**Figure 4.3** Mass spectrum of de-sialylated IgA1 hinge glycopeptide. The spectrum has become simplified due to the removal of all the sialic acid residues, leaving a number of discrete peaks (with additional sodiated and potassiated and incompletely de-sialylated species). In this example the observed peaks 5302 (calculated m/z 5304), 5140 (calculated m/z 5140), 5018 (calculated m/z 5021), 4978 (calculated m/z 4978), 4937 (calculated m/z 4937), 4775 (calculated m/z 4775), and 4572 (calculated m/z 4573) correspond to the asialyl-species 550,540,620,530,440,430,and 330 respectively.

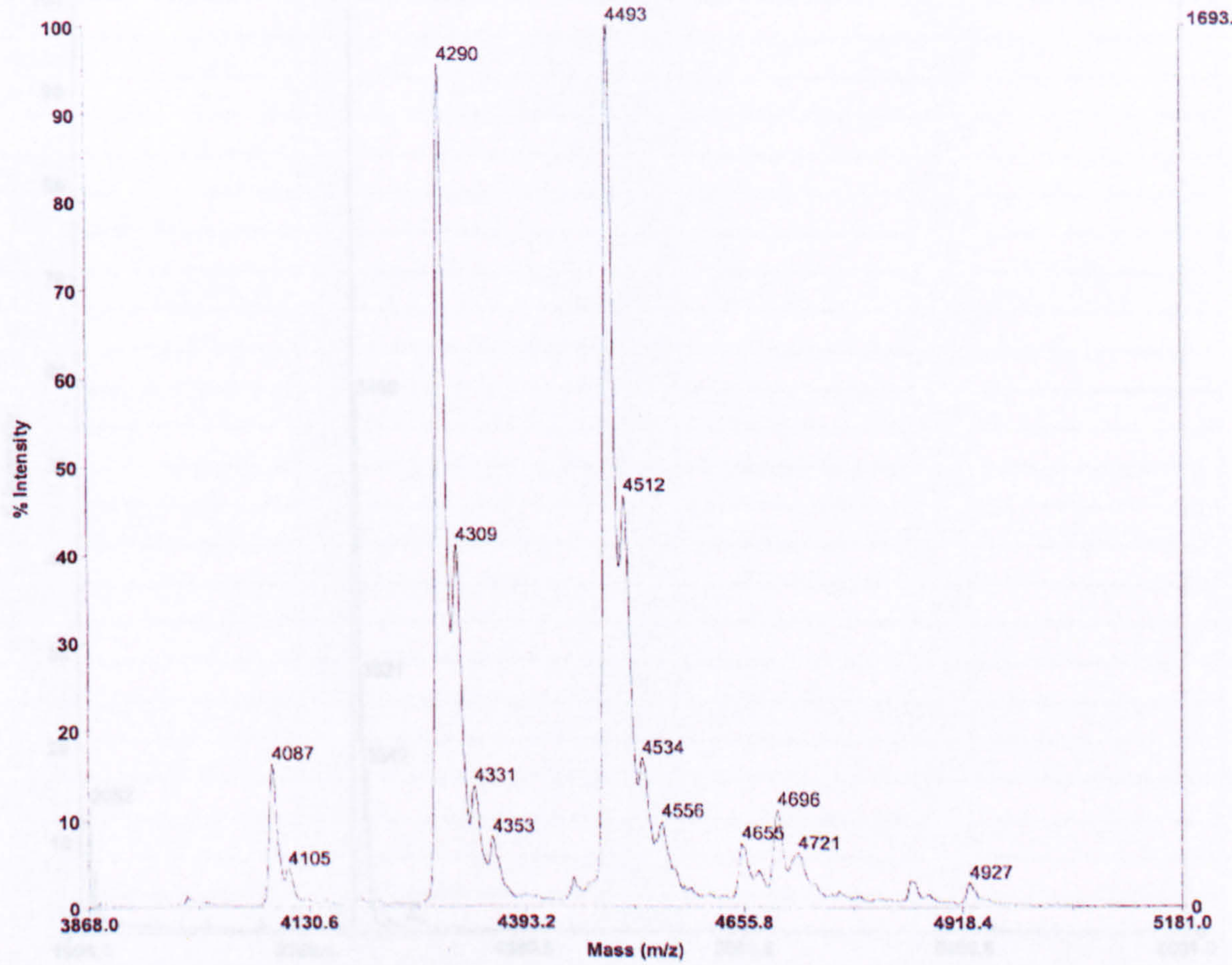



**Figure 4.4** Mass spectra from the de-sialylated and de-galactosylated hinge glycopeptide with a number of distinct peaks reflecting the presence of the asialo-, agalacto- forms of the hinge glycopeptide. Peak 4087 (calculated m/z 4085) corresponds to the 300 glycoform while peaks 4290 (calculated m/z 4289) and 4493 (calculated m/z 4492) correspond to the 400 and 500 glycoforms respectively. The small peak assigned to 4696 (calculated m/z 4695) corresponds to the 600 species. The remaining observed peaks correspond to sodiated, postassiated, or incompletely de-glycosylated species.

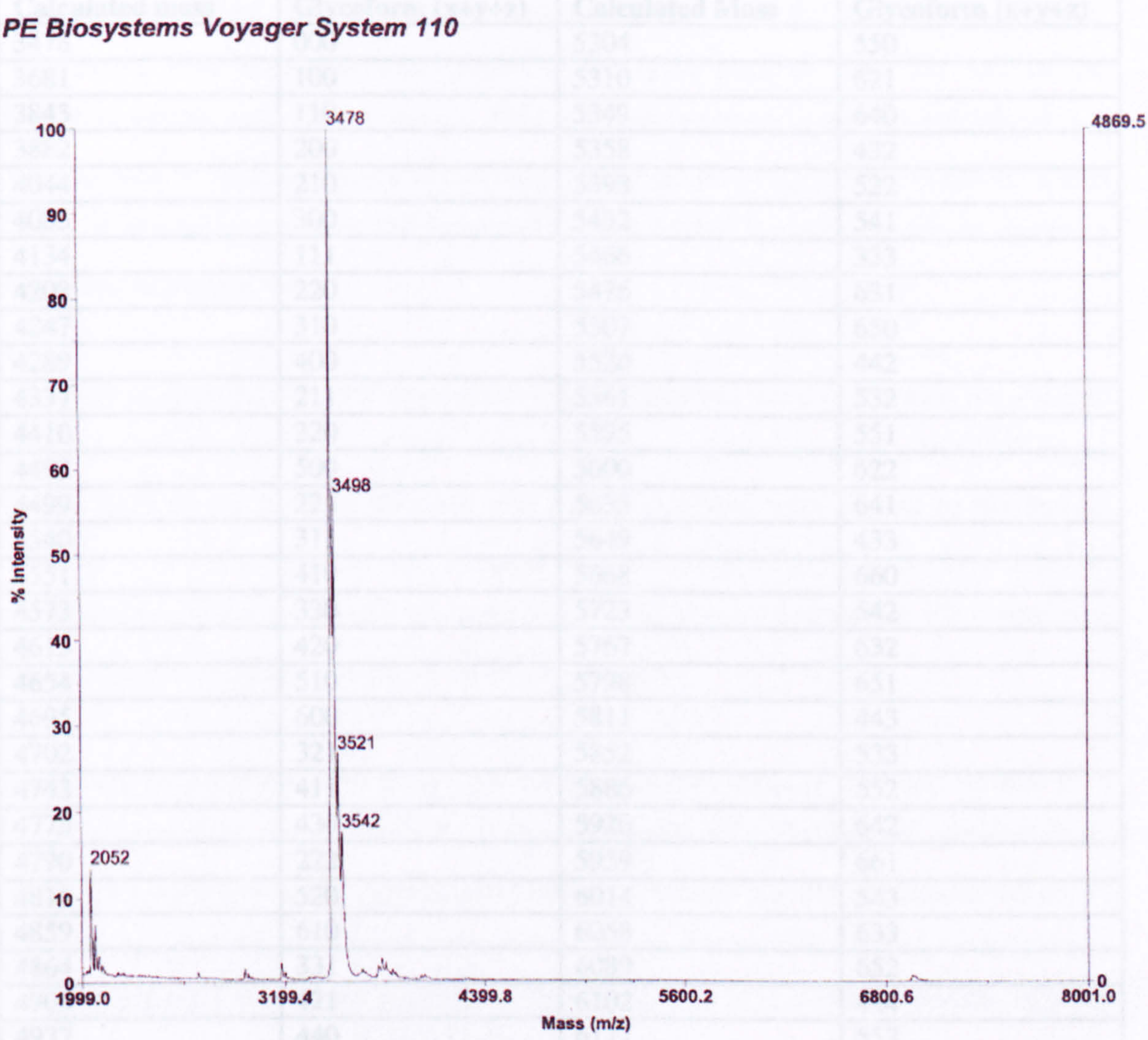



**Figure 4.5** Mass spectrum of the totally de-glycosylated normal IgA1 hinge glycopeptide showing the single peak with a mass of 3478, which represents the 33 amino acid chain present in the tryptic digest of the reduced/pyridylated IgA1 to which the O-linked glycans are normally attached. The smaller peaks labelled are single, double, and treble sodiated species identified by the mass spectrometer of the same species.

| Calculated mass | Glycoform (x+y+z) | Calculated Mass | Glycoform (x+y+z) |
|---|---|---|---|
| 3478 | 000 | 5304 | 550 |
| 3681 | 100 | 5310 | 621 |
| 3843 | 110 | 5349 | 640 |
| 3882 | 200 | 5358 | 432 |
| 4044 | 210 | 5398 | 522 |
| 4085 | 300 | 5432 | 541 |
| 4134 | 111 | 5466 | 333 |
| 4208 | 220 | 5476 | 631 |
| 4247 | 310 | 5507 | 650 |
| 4289 | 400 | 5520 | 442 |
| 4337 | 211 | 5561 | 532 |
| 4410 | 320 | 5595 | 551 |
| 4492 | 500 | 5600 | 622 |
| 4499 | 221 | 5635 | 641 |
| 4540 | 311 | 5649 | 433 |
| 4551 | 410 | 5668 | 660 |
| 4573 | 330 | 5723 | 542 |
| 4613 | 420 | 5767 | 632 |
| 4654 | 510 | 5798 | 651 |
| 4695 | 600 | 5811 | 443 |
| 4702 | 321 | 5852 | 533 |
| 4743 | 411 | 5886 | 552 |
| 4775 | 430 | 5926 | 642 |
| 4790 | 222 | 5959 | 661 |
| 4816 | 520 | 6014 | 543 |
| 4859 | 610 | 6058 | 633 |
| 4864 | 331 | 6089 | 652 |
| 4905 | 421 | 6102 | 444 |
| 4937 | 440 | 6177 | 553 |
| 4945 | 511 | 6217 | 643 |
| 4978 | 530 | 6250 | 662 |
| 4993 | 322 | 6307 | 544 |
| 5021 | 620 | 6380 | 653 |
| 5066 | 431 | 6468 | 554 |
| 5107 | 521 | 6508 | 644 |
| 5140 | 540 | 6541 | 663 |
| 5150 | 611 | 6671 | 654 |
| 5155 | 332 | 6759 | 555 |
| 5185 | 630 | 6832 | 664 |
| 5196 | 422 | 6962 | 655 |
| 5228 | 441 | 7123 | 665 |
| 5270 | 531 | 7414 | 666 |

**Table 4.1** Table of theoretical IgA1 hinge glycopeptide calculated masses and their corresponding glycoforms. x=number of N-Acetyl galactosamine (GalNAc) residues, y=galactose (Gal) residues and z=the number of N-acetyl neuraminic acid (NANA) residues in each glycoform, the difference between the different glycoforms being due to the varying number of oligosaccharides. The masses of NANA, GalNAc, and Gal are 291, 203 and 162 respectively.

## 4.4 Normal spectra

Having established that the spectra obtained so far were those of the IgA1 HGP glycoforms, the technique was applied to the IgA1 purified from the sera of the different patient and control groups. This included work on myeloma, HSP/myeloma sera as well as paired sera and breast milk from two lactating normal controls. There were 24 normal serum samples processed for IgA1 HGP analysis. A sample spectrum is shown in **Figure 4.6**.

In order to determine signal reproducibility, 10 analyses were performed on a single spot of a HGP pool that had been dried together with matrix onto a MALDI target plate. Each of the profiles obtained was then normalised with respect to the largest peak present in the spectrum and the peak area of individual glycoforms calculated as a percentage variation around its mean value. The spectra and values for percentage peak area were identical in the 10 different readings.

Similarly 10 analyses were performed on 10 different spots of a single sample of a HGP pool and the results similarly analysed (**Figure 4.7**). The spectra obtained from the pools, exhibited high reproducibility with a mean coefficient of variance (C of V) of 10% (range 5-15%) (based on the percentage peak area of each peak as a function of the total peak area of the spectrum). This enabled us to confidently use the data for the relative quantification of the different glycan species present in different patient and control groups. Furthermore, one serum sample which was split into 4 and processed in parallel as 4 individual samples from start to finish, were analysed individually to assess reproducibility throughout the preparation and analysis of the HGP. This also demonstrated good reproducibility between the spectra obtained (**Figure 4.8**). The mean percentage peak areas and the co-efficient of variance were calculated for each sample and the major peaks were found to have a mean C of V of 14% (ranging between 3.8 to 24 %).

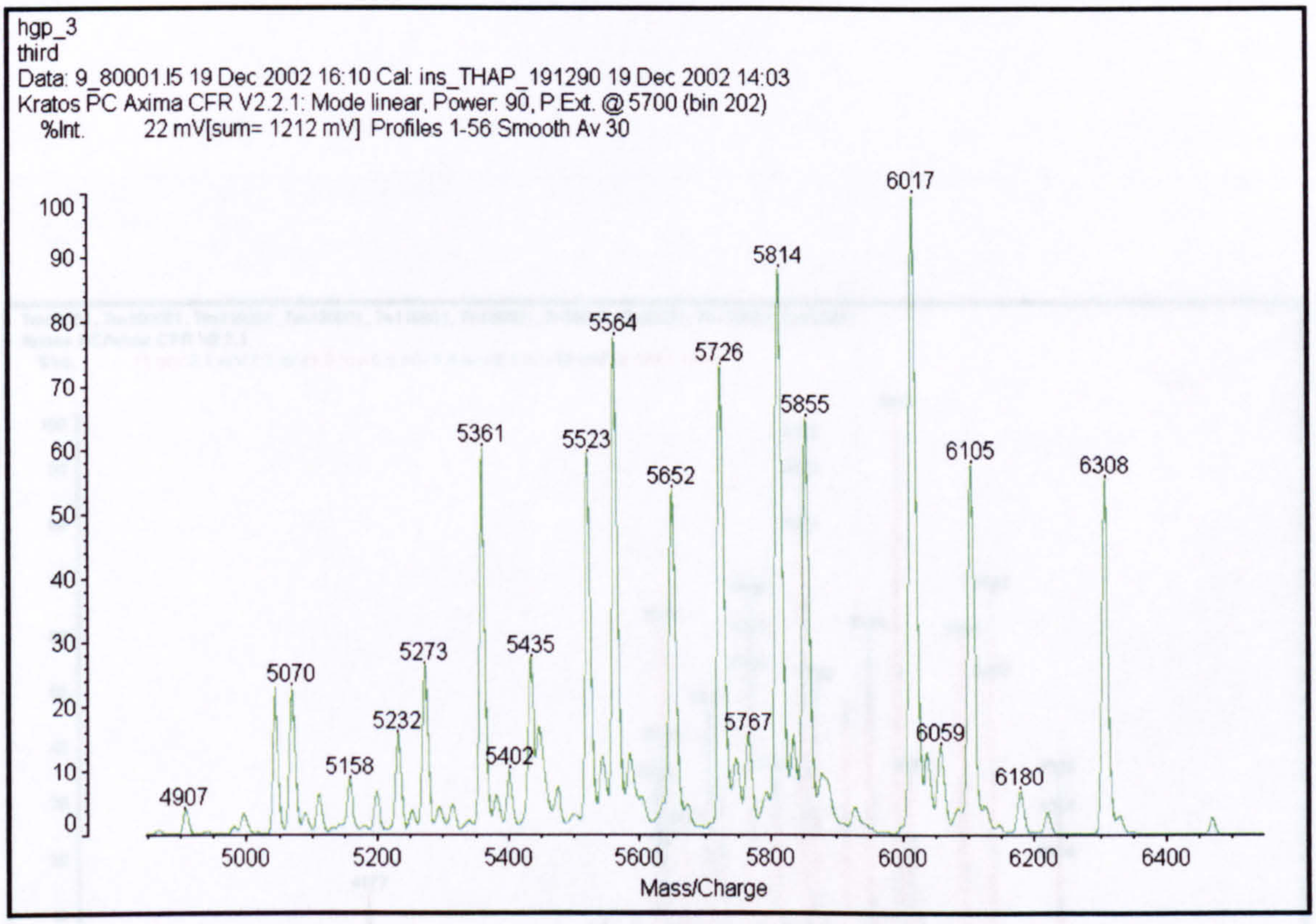



**Figure 4.6** A mass spectrum showing the peaks obtained from serum IgA1 hinge glycopeptide from a normal subject.

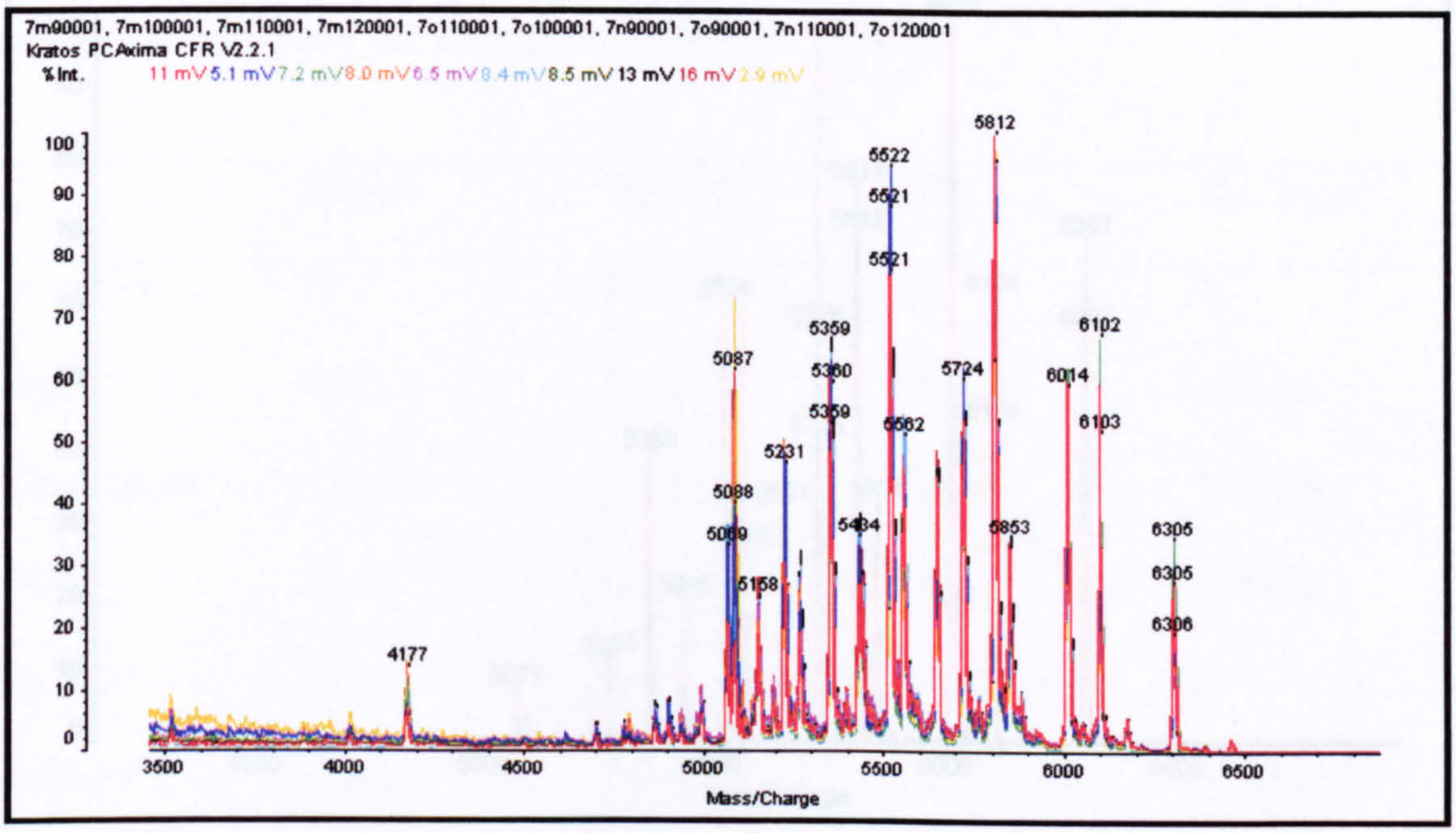



**Figure 4.7** Superimposed spectra from a single IgA1 HGP sample spotted 10 times onto a MALDI target plate for analysis. The spectra demonstrate that the peaks are identical despite being split and analysed separately. The percentage peak area as a percentage of the total peak area for each spectrum was also comparable.

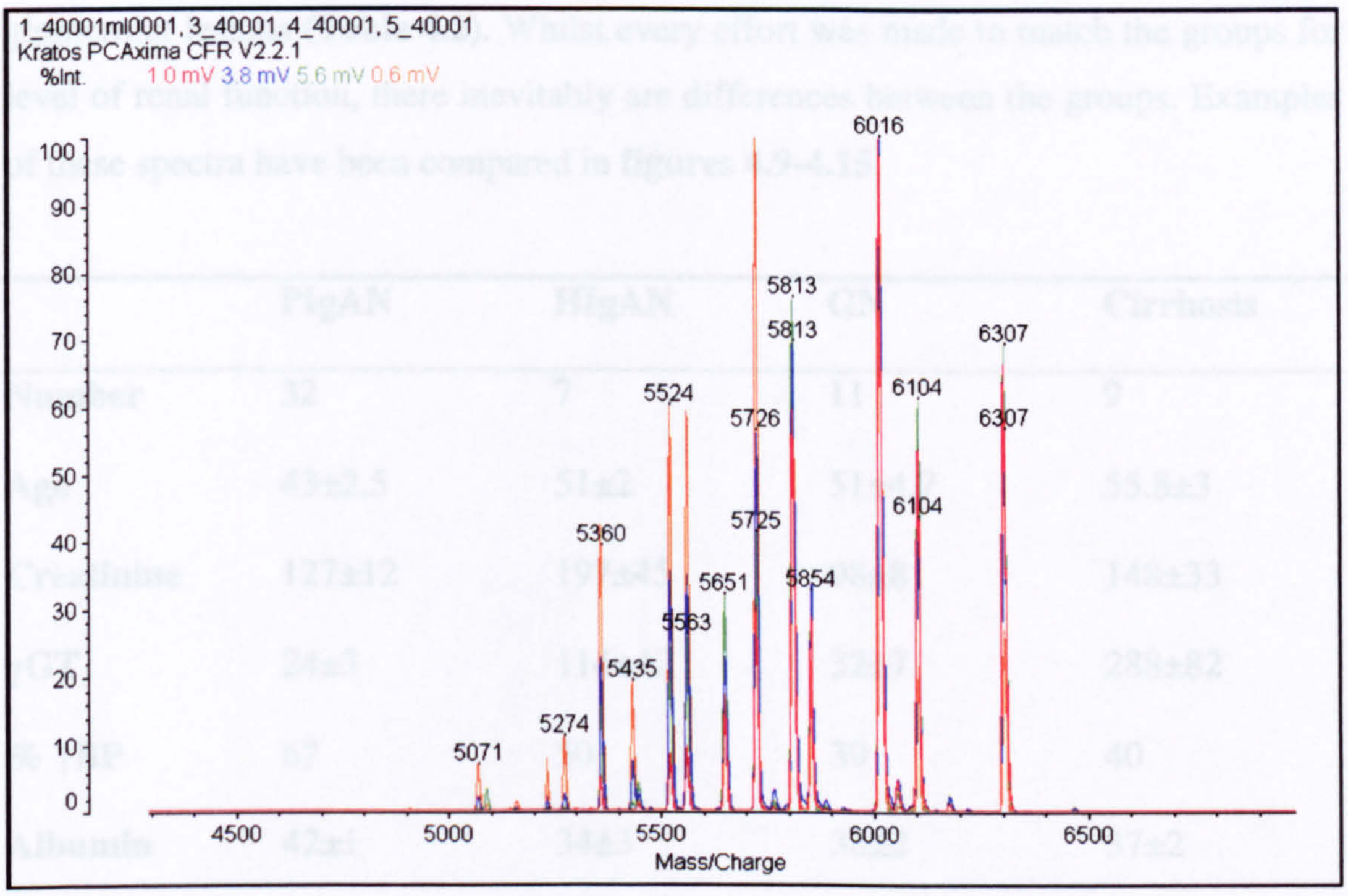



**Figure 4.8** IgA1 HGP superimposed spectra from single sample of normal serum processed 4 times separately.

## 4.5 Pathological spectra

Serum samples from patients and their patient controls were also analysed using MALDI ToF-MS. There were 32 samples from patients with primary IgAN, 11 samples from patients with non-IgA glomerulonephritis, 7 with hepatic IgAN, 9 with cirrhosis of the liver with no evidence of glomerular disease, 2 samples from patients with HSP secondary to myeloma and 4 patients with myeloma and no associated renal glomerular lesions (**Table 4.2**). Whilst every effort was made to match the groups for level of renal function, there inevitably are differences between the groups. Examples of these spectra have been compared in **figures 4.9-4.15**.

| | **PIgAN** | **HIgAN** | **GN** | **Cirrhosis** |
|---|---|---|---|---|
| **Number** | 32 | 7 | 11 | 9 |
| **Age** | 43±2.5 | 51±2 | 51±4.7 | 55.8±3 |
| **Creatinine** | 127±12 | 197±45 | 98±8 | 148±33 |
| **γGT** | 24±3 | 114±42 | 32±7 | 288±82 |
| **% ↑BP** | 67 | 50 | 39 | 40 |
| **Albumin** | 42±1 | 34±3 | 38±2 | 37±2 |
| **Uprotein (g/d)** | 0.9±0.3 | 1.6±0.8 | 1.3±0.6 | 0.2±0.01 |

**Table 4.2** This table summarises the clinical parameters measured in the main patient groups. Results are the mean values for each group ± SEM. There were no statistically significant differences in the age of the patients in the different groups. Statistical analyses were done between PIgAN and HIgAN, PIgAN and GN, and HIgAN and cirrhosis. Blood pressure, urinary protein excretion and serum creatinine levels were also comparable between the groups with no statistically significant differences found. Patients with PIgAN had statistically higher albumin levels compared with both HIgAN and patients with GN (p values $< 0.007$ and 0.03 respectively). γGT levels were not statistically different between the study and control groups. Abbreviations: PIgAN: patients with primary IgAN, GN: patients with renal glomerular disease other than IgAN, HIgAN: hepatic IgAN, % ↑BP. Percentage of patients with hypertension in each group, Uprotein, urinary protein excretion per day

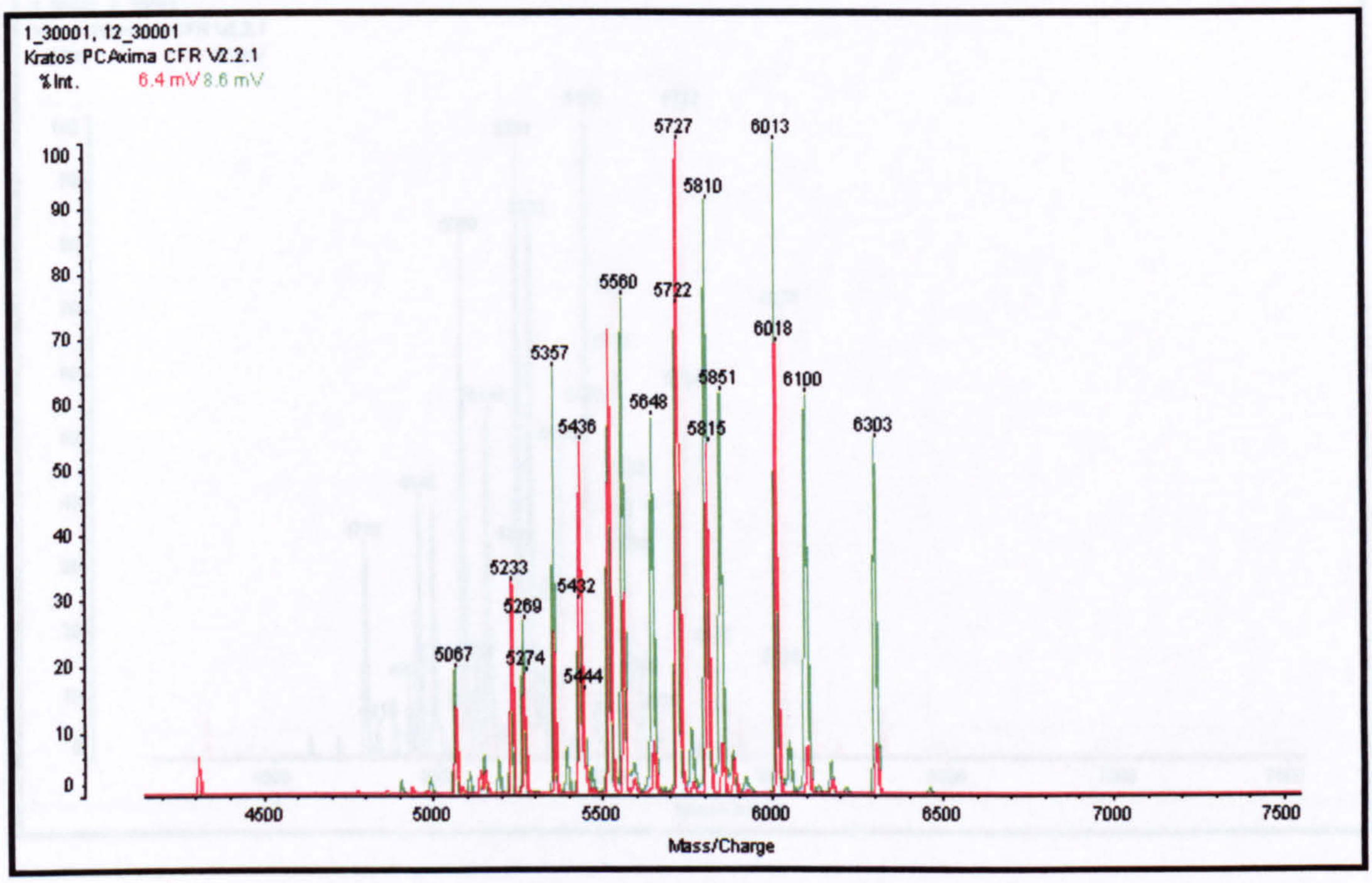



**Figure 4.9** Superimposed sample of IgA1 HGP spectrum from a normal subject (green) and patient with primary IgAN (red). This example demonstrates the difference between more complex glycoforms with higher mass/charge (m/z) values and those with relatively reduced glycan content. A shift towards the lower m/z is evident in the patient with IgAN as compared to the normal individual in this example.

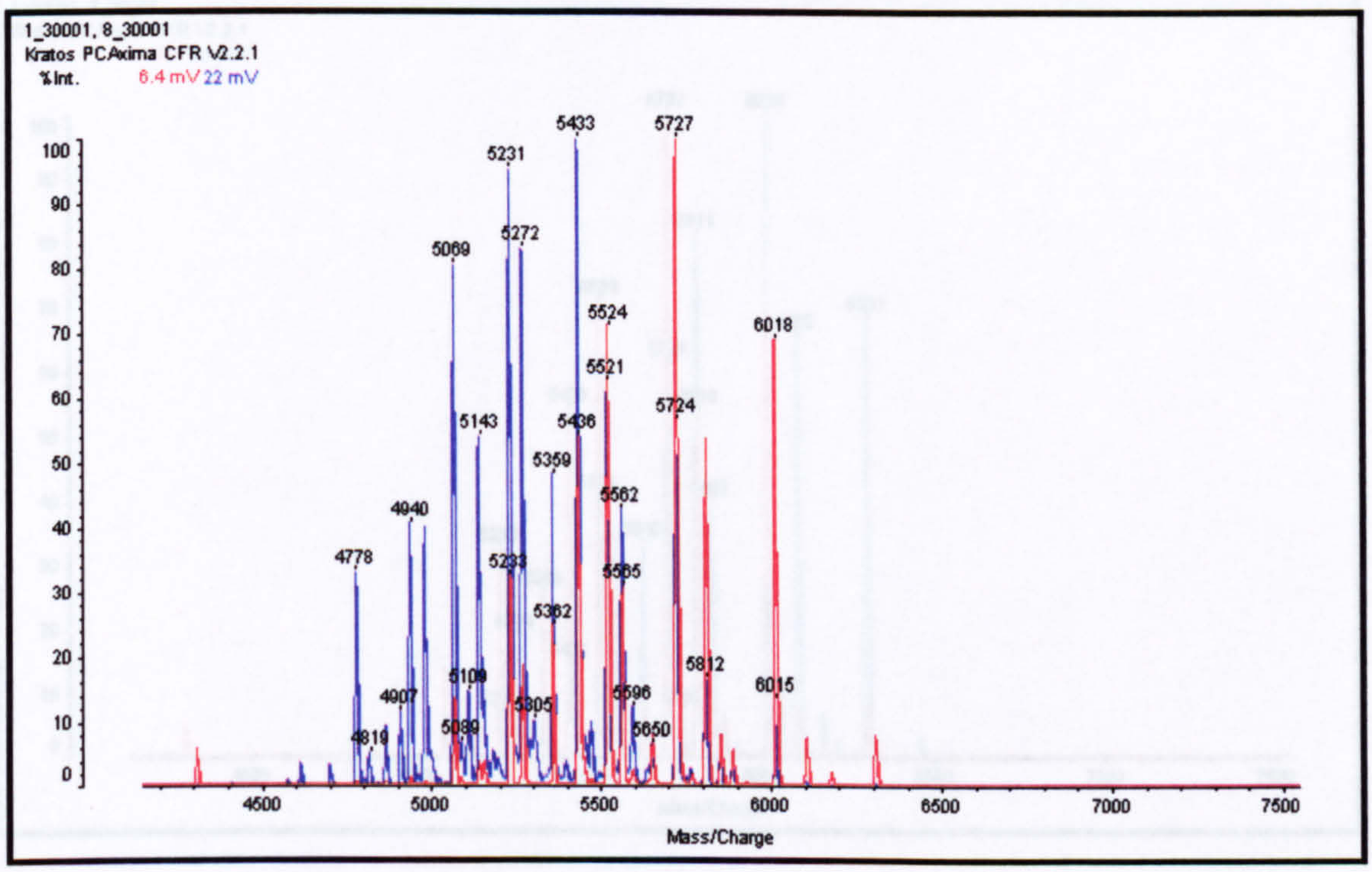



**Figure 4.10** IgA1 HGP spectra from a patient with primary IgAN (red) and hepatic IgAN (blue) superimposed to demonstrate differences in glycoform prevalence. In this example the patient with HIgAN appears to have a striking shift in the glycoforms with a lower mass/charge compared to the patient with primary IgAN.

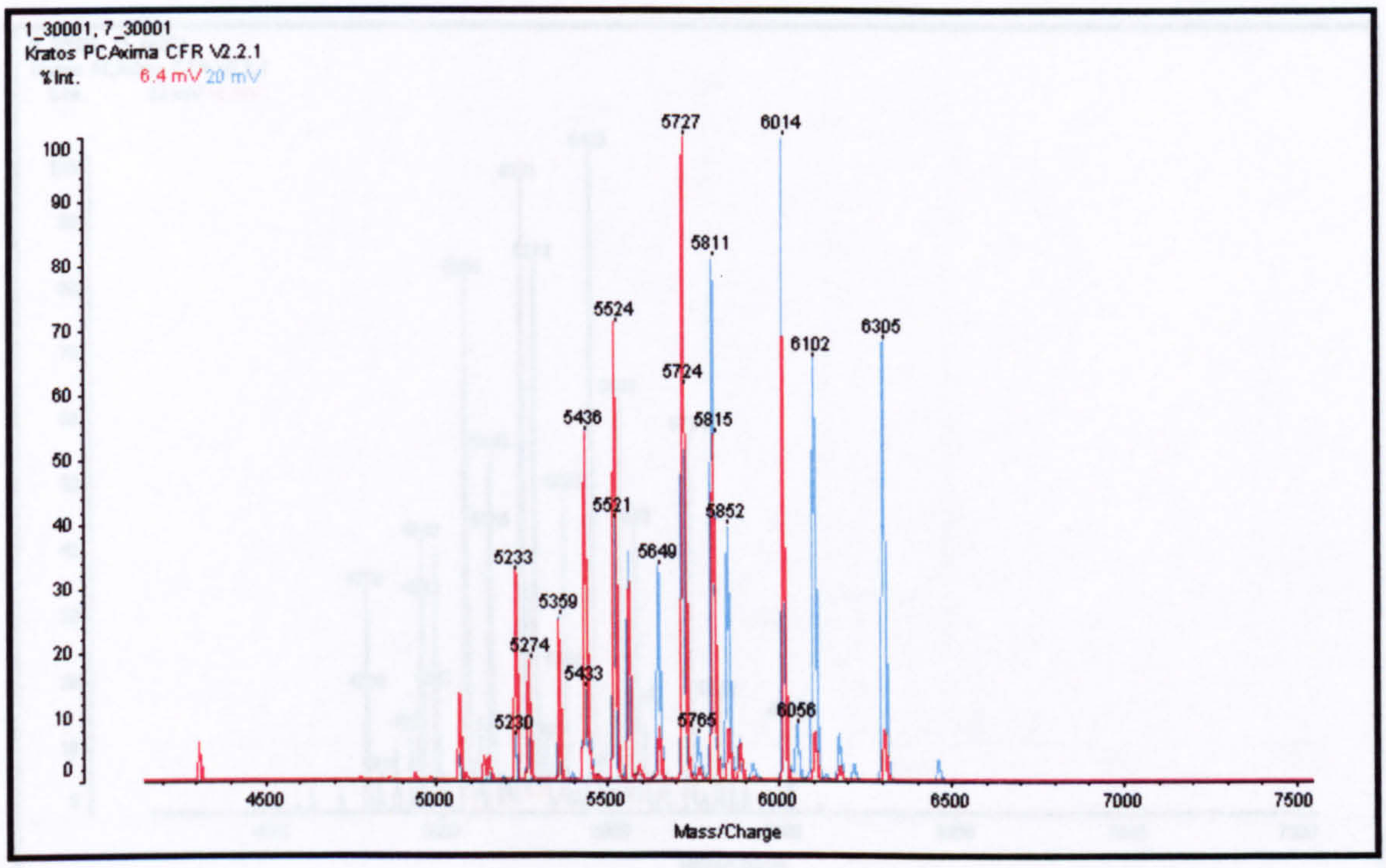



**Figure 4.11** Superimposed spectra from IgA1 HGP sera from a patient with primary glomerulonephritis of non-IgA type (blue) versus one from a patient with primary IgAN (shown in red). The spectrum from the patient with PIgAN has a greater preponderance of the lower mass/charge glycoforms compared with its control.

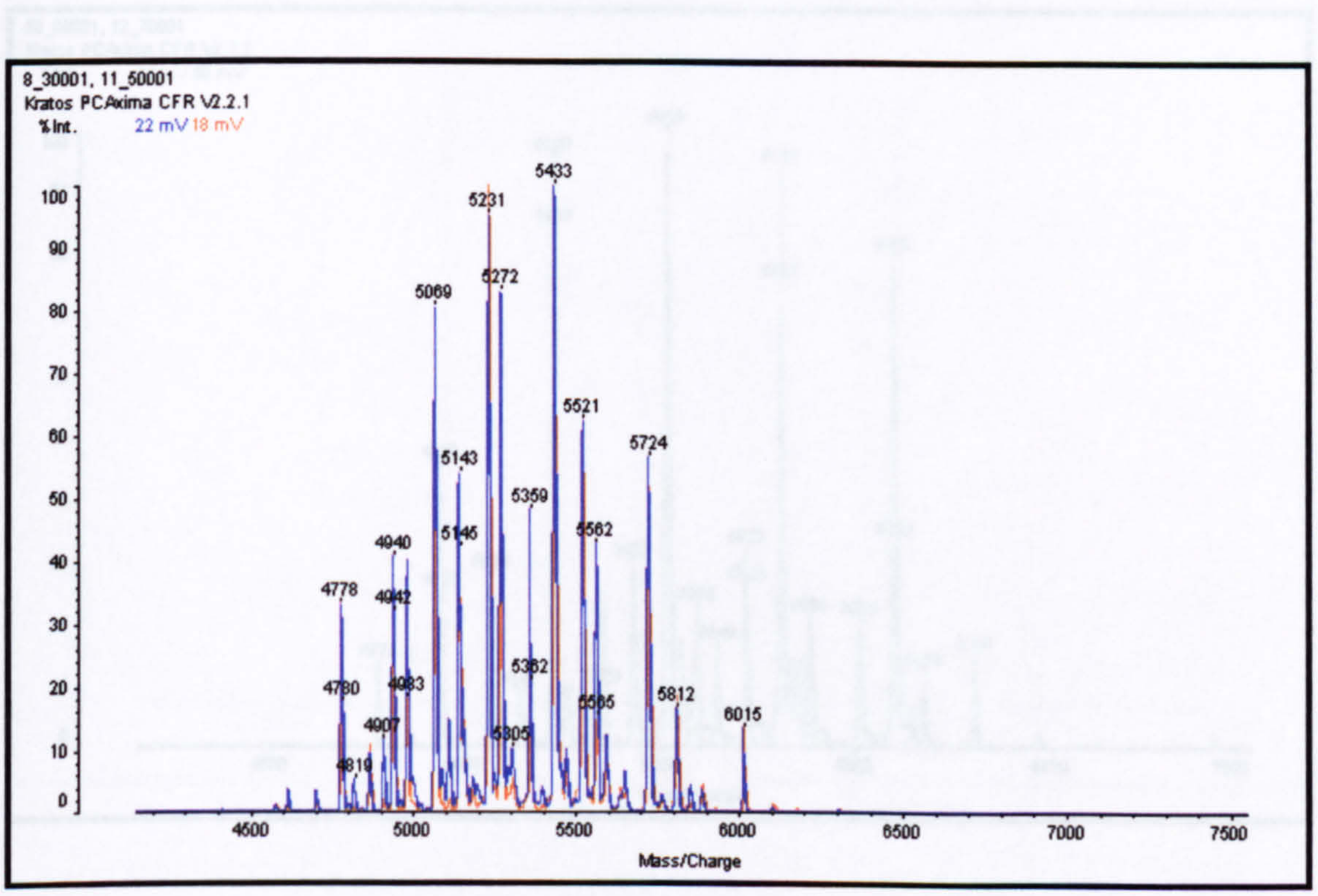



**Figure 4.12** Superimposed spectra of a patient with hepatic IgAN (blue trace) and another with cirrhosis (orange trace) showing no difference in spectra between the two patients. This suggests that the glycoforms present in these examples have very similar glycan content to each other.

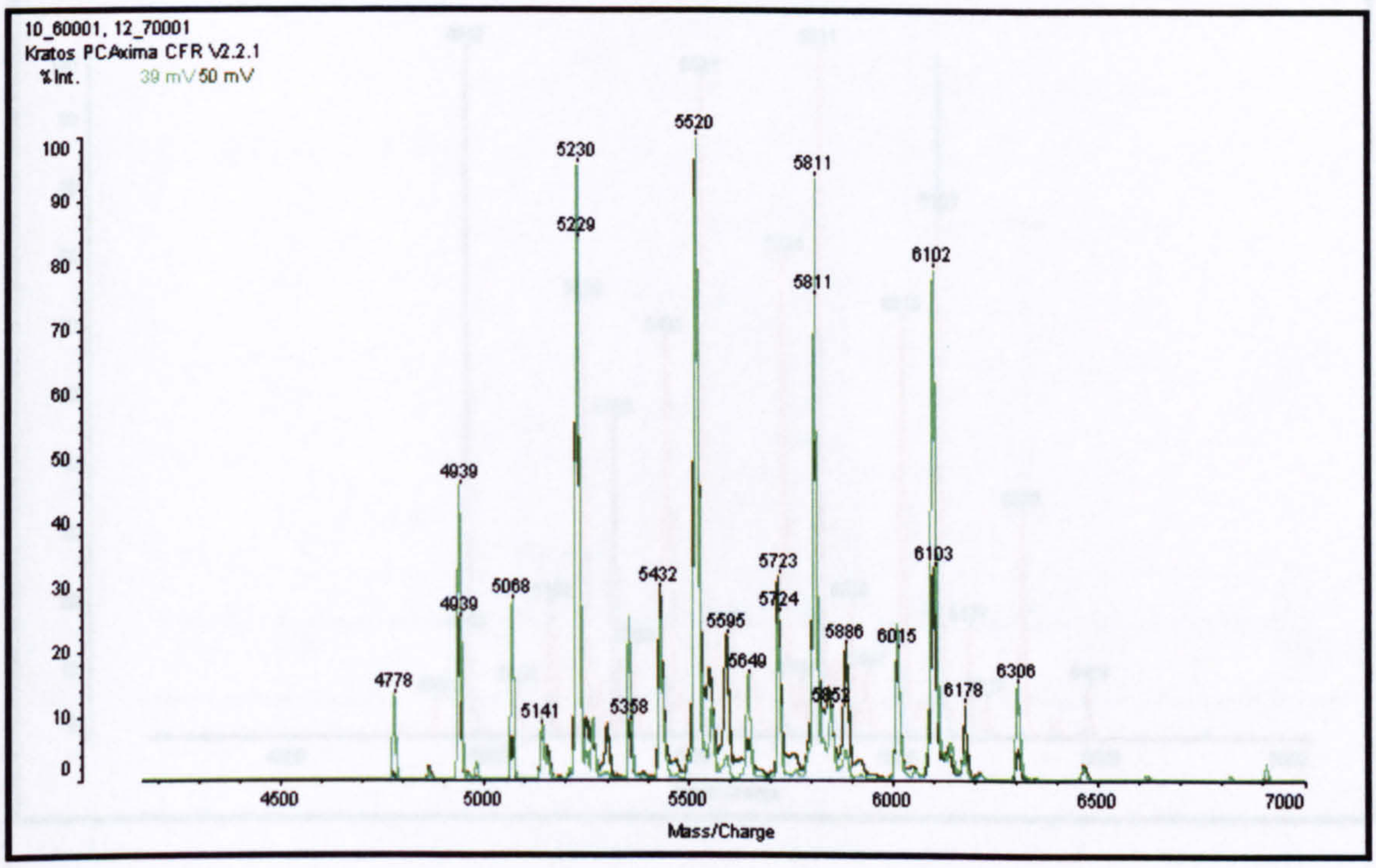



**Figure 4.13** Overlaid IgA1 hinge glycopeptide spectra from two patients with Henoch-Schönlein Purpura secondary to IgA1 myeloma. The spectra have a different range of glycoforms as would be expected from two different monoclonal expansions of the B cells producing IgA1 protein.

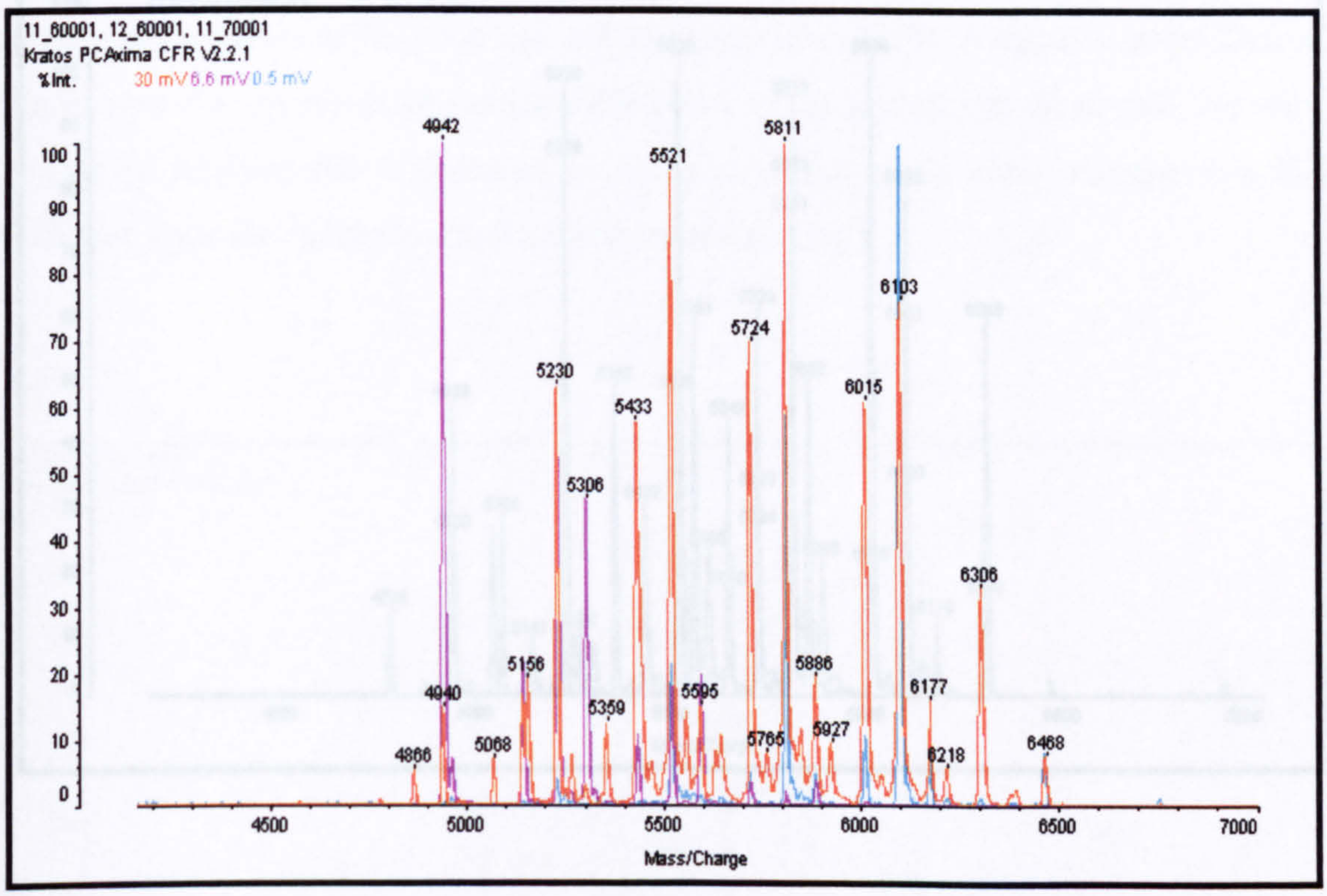



**Figure 4.14** Superimposed IgA1 HGP spectra from three patients with myeloma with no nephropathy. The 3 spectra are completely different in their composition, reflecting the distinct monoclonal production of myeloma proteins.

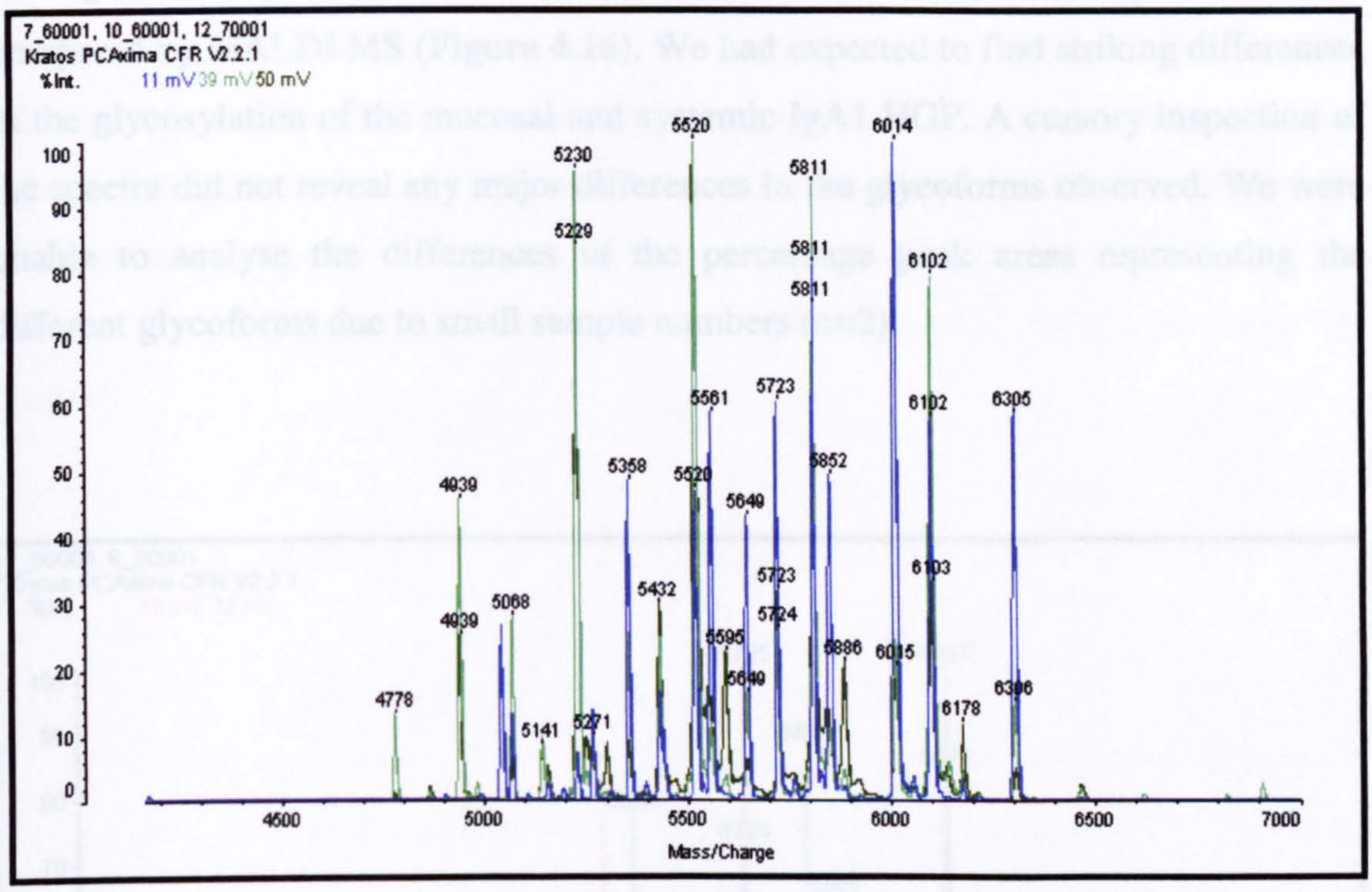



**Figure 4.15** Spectra from IgA1 hinge glycopeptide of normal serum (navy trace) compared with that of a patient with Henoch-Schönlein Purpura secondary to IgA1 myeloma (green trace). The patient in this example with nephropathy demonstrates a shift towards the lower mass/charge and suggesting the presence of predominantly under glycosylated glycoforms as compared with the normal control.

## 4.6 Spectra obtained from mucosal IgA1 HGP

The IgA1 from paired sera and breast milk of two healthy lactating women was compared by MALDI MS (**Figure 4.16**). We had expected to find striking differences in the glycosylation of the mucosal and systemic IgA1 HGP. A cursory inspection of the spectra did not reveal any major differences in the glycoforms observed. We were unable to analyse the differences in the percentage peak areas representing the different glycoforms due to small sample numbers (n=2).

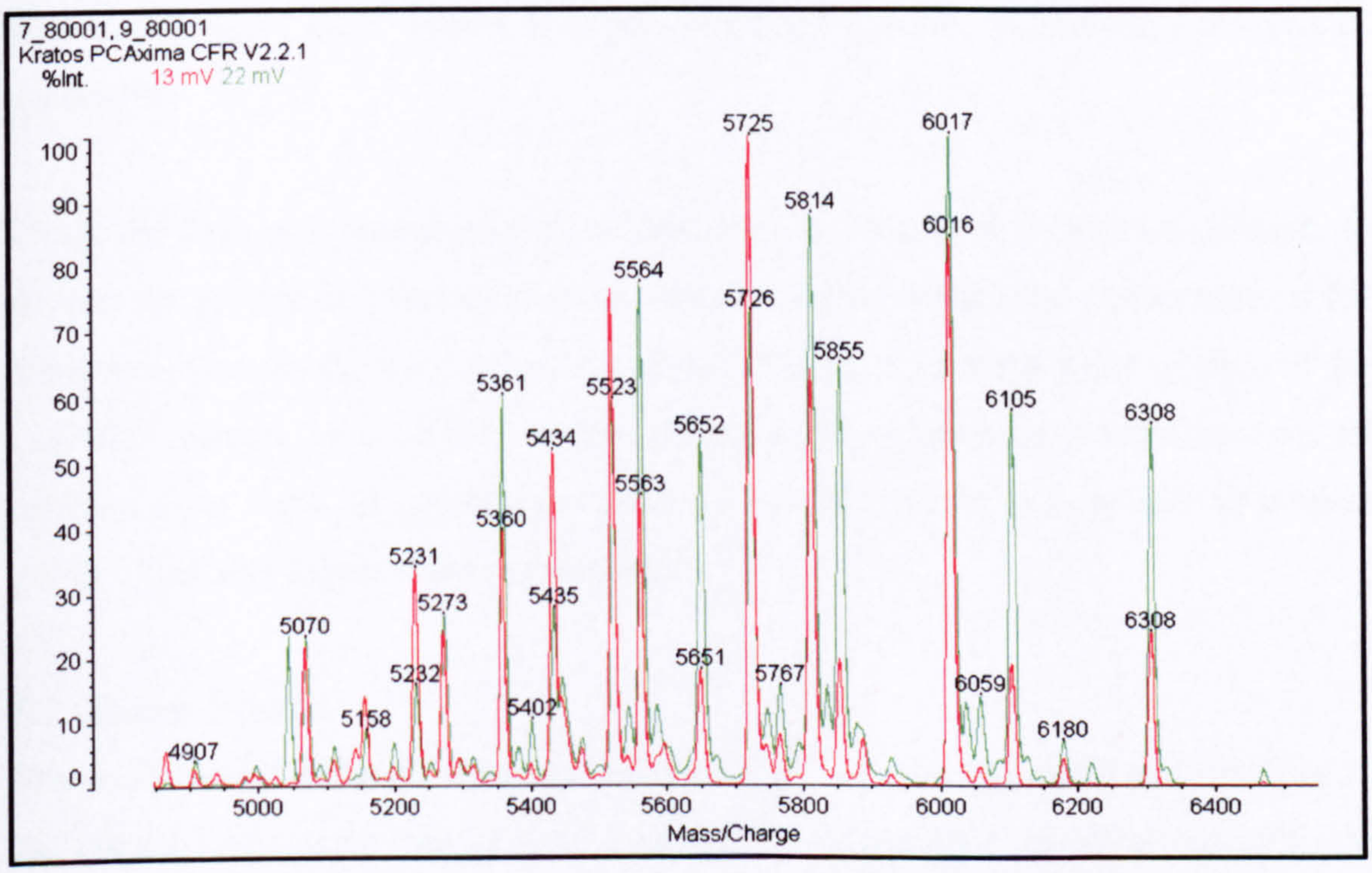



**Figure 4.16** Spectra comparing the traces obtained from paired samples of normal serum and breast milk IgA1 HGP. The green trace represents the glycoforms of the IgA1 HGP of the normal serum, overlaid with the spectrum from breast milk IgA1 HGP in red.

## 4.7 Summary

The figures presented in this chapter are a culmination of the technique used to purify and analyse the IgA1 hinge peptide and its glycans by MALDI-ToF mass spectrometry. These spectra demonstrate the unrivalled quality achieved in this study. The spectra are baseline resolved and the peaks form discrete entities allowing accurate analyses of the carbohydrates in the HGP as well as comparisons between the different patient and control groups. Having presented the IgA1 HGP spectra in this chapter, the statistical analysis of the peaks will be carried out in the following chapter.

# Chapter 5: Analyses of the IgA1 hinge glycopeptide spectra and their significance

## 5.1. Introduction

The complexity and variety of the glycoforms obtained by MALDI mass spectrometry present a daunting array of results requiring meaningful statistical analyses. Previous studies of IgA1 glycosylation by mass spectrometry have relied on comparing peak areas to a nominal peak, allowing at best a descriptive presentation of the findings. Also the poor baseline resolution of the spectra obtained by other groups has made comparisons between patient and control groups unreliable. Having obtained very high quality IgA1 HGP spectra, it is now possible to tackle the statistical analysis of the results.

Using the Java programme output, as described in chapter 3, it became possible to process the spectra in a variety of ways, namely differences in total glycan number for the three different oligosaccharides found in the HGP, percentage glycosylation of the GalNAc residue, rank order of the different glycoforms and identification of differences in terms of glycoform omissions or additions in each patient or control group. This data is presented in this chapter.

## 5.2 Glycan content

The incidence of different numbers of GalNAc, galactose, and sialic acid residues in the patient and control groups were analysed by calculating the mean value for the percentage peak area of all the spectra in each group for each glycoform. The mean percentage peak area for all species containing different numbers of oligosaccharides found in each patient and control group was calculated. The mean percentage peak area for each group was then compared using the Mann Whitney U test and Bonferroni correction. **Table 5.1** summarises the findings of the glycan analyses performed by presenting the mean number of sugar residues per IgA molecule.

| | MEAN NANA NUMBER | MEAN GAL NUMBER | MEAN GALNAC NUMBER |
|---|---|---|---|
| **Normal** | 2.42 | 3.6 | 4.52 |
| **PIgAN** | 1.94‡ | 3.4 | 4.43‡ |
| **GN** | 1.99 | 3.4 | 4.34‡ |
| **HIgAN** | 1.35‡ | 3.7 | 4.31‡ |
| **Cirrhosis** | 1.55‡ | 3.6 | 4.33 |
| **Myeloma** | 2.1 | 4.0‡ | 4.32‡ |
| **HSP/myeloma** | 2.1 | 3.9 | 4.26 |
| **Breast Milk** | 2.1 | 3.9 | 4.29 |

**Table 5.1** Mean number of sugar residues per patient and control groups. ‡ denotes the presence of a statistically significant difference ($p<0.05$) with respect to normal when applying the Mann Whitney U test and the Bonferroni correction to the results. The numbers represent the average glycan residues per IgA molecule in the different groups. NANA=Sialic Acid, Gal=Galactose, GALNAC=N-acetylgalactosamine

## 5.2.1 GalNAc

The literature suggests that there are 9 potential sites for GalNAc residues via which they can attach to the polypeptide backbone of the IgA1 HGP. Three of these are presumed to be fully occupied and the other 2 partially occupied (**figure 2.5**) (Mattu et al 1998). We identified a 6$^{th}$ GalNAc substitution present in all our patient and control groups (except for patients with HSP secondary to myeloma). Our findings suggest that this hexa-substitution is a universal phenomenon.

Previous studies of IgA glycosylation in IgAN have found no difference in the GalNAc content of patients with primary IgAN and healthy controls. Our detailed comparisons between the prevalence of the number of GalNAc residues in each group have highlighted a number of differences. **Figure 5.1** summarises these findings in a bar chart. Each patient and control group is represented by a bar measuring the mean percentage peak area of each glycan. The mean number of GalNAc residues found in each groups is shown in **table 5.1.** Whilst the mean GalNAc number for normal controls was 4.52, by comparison patients with PIgAN, other GNs, HIgAN, and myeloma had significant reduction in their mean GalNAc content.

**Figure 5.2** shows a scatter graph of the mean percentage peak area of the 3 GalNAc species in the different subjects. Species containing only 3 GalNAc residues constitute a small proportion of the glycoforms present in normal individuals (**Figure 5.1**). The mean value for this group was 1.9% ± 2.3 (standard deviation of the mean). By comparison, patients with primary and hepatic IgAN had elevated means of 3.7% ± 4.0 and 7.1% ± 6.5 respectively. This achieved statistical significance with $p<0.04$ for PIgAN and $p<0.02$ in HIgAN using the MWU. The mean values for the myeloma group and the results from mucosal IgA (samples of breast milk from normal controls) were similar to normals. Even though the mean for patients with HSP secondary to myeloma was strikingly lower than any of the other groups, the small number of patients (n=2) does not allow any meaningful statistical comparisons to be made with normal controls.

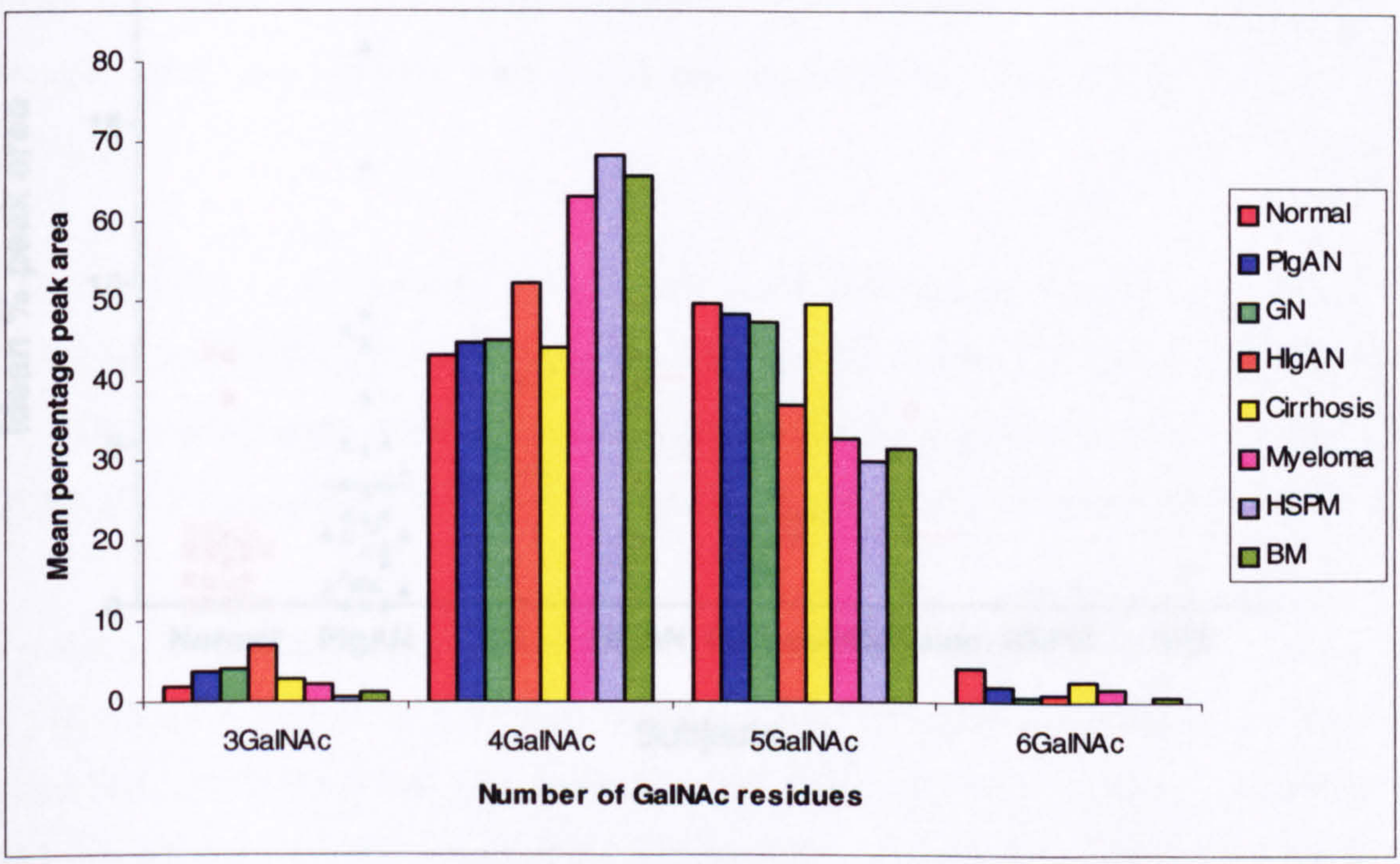



**Figure 5.1** Bar chart showing a comparison between the GalNAc numbers in the different patient groups.

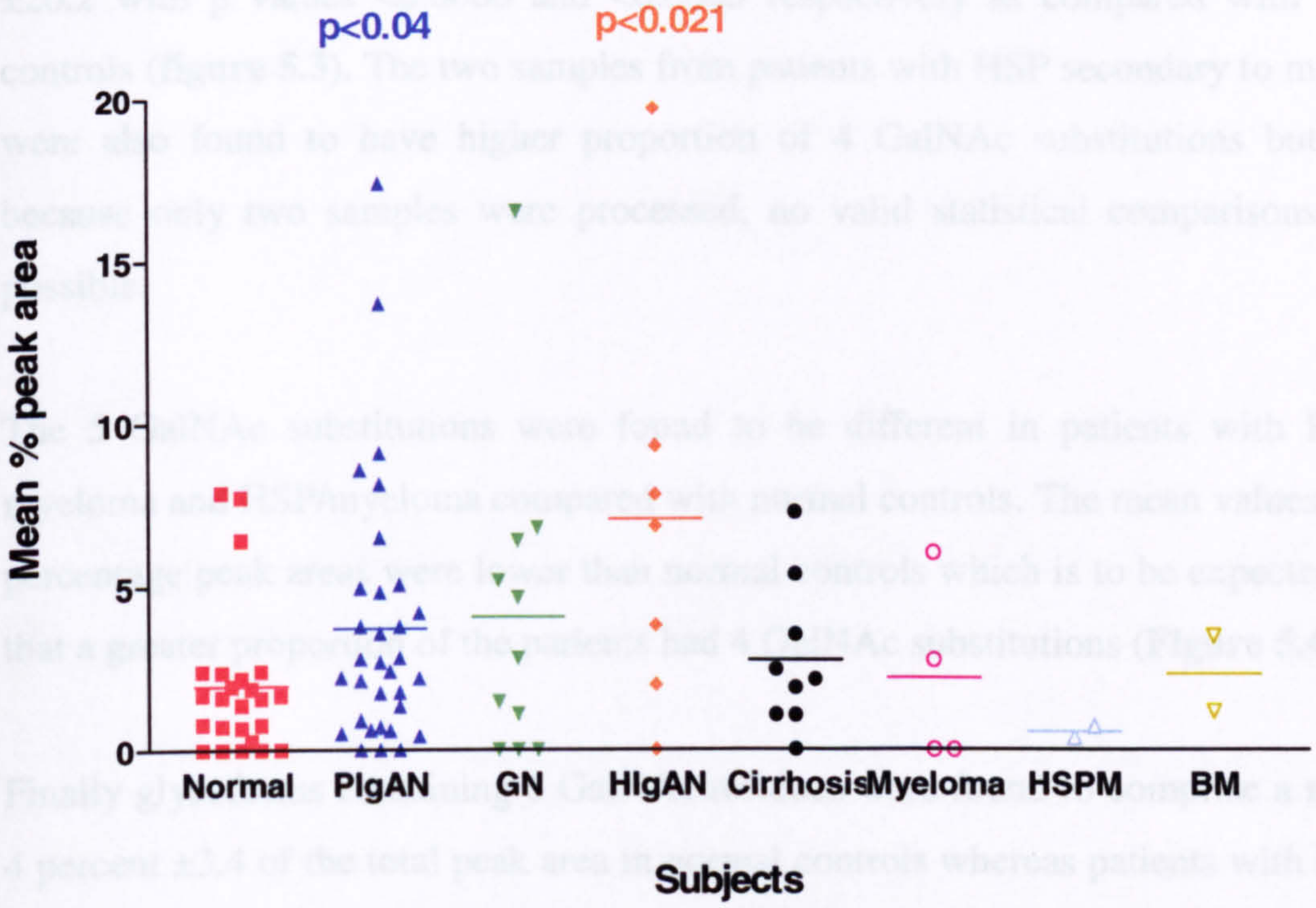



**Figure 5.2** Scatter graph showing the difference in percentage peak area of glycoforms with 3 GalNAc in the study groups. Both patients with primary and hepatic IgAN had a greater number of glycoforms with this species present.

4 GalNAc substitutions were more common than 3 GalNAc in all the groups and in breast milk with a mean value of 43.4% ±5.3 in normal controls. Like the 3 GalNAc species, there were statistically significant differences between patients with HIgAN (but not PIgAN) and myeloma as compared with the normal controls. The mean percentage peak area for HIgAN and myeloma were calculated as 52.3 ±9.9 and 63.2 ±20.2 with p values <0.0066 and <0.0065 respectively as compared with normal controls (**figure 5.3**). The two samples from patients with HSP secondary to myeloma were also found to have higher proportion of 4 GalNAc substitutions but again, because only two samples were processed, no valid statistical comparisons where possible.

The 5 GalNAc substitutions were found to be different in patients with HIgAN, myeloma and HSP/myeloma compared with normal controls. The mean values for the percentage peak areas were lower than normal controls which is to be expected given that a greater proportion of the patients had 4 GalNAc substitutions (**Figure 5.4**).

Finally glycoforms containing 6 GalNAc residues were found to comprise a mean of 4 percent ±3.4 of the total peak area in normal controls whereas patients with PIgAN, other GN, and HIgAN had lesser values at 2.0 ±1.6 (p<0.011), 0.8 ±0.97 (p<0.0006), and 0.9 ±1.3 (p<0.0066) respectively (**figure 5.5**). Patients with myeloma showed a trend towards lesser 6 GalNAc substitutions that did not achieve statistical significance and no 6 GalNAc containing glycoforms were identified in the 2 patients with HSP myeloma. In contrast to systemic IgA1, mucosal IgA1 from breast milk had a lower proportion of IgA1 glycoforms containing 6 GalNAc residues, the statistical significance of this does remain doubtful due to the small number of samples (n=2).

Having compared the different patient and control groups, the Bonferroni statistical correction was applied to the obtained p values in order to modify the significance of the results by taking into account the multiple comparisons between the different subject groups. Following this manipulation there was no statistically significant difference between the mean percentage peak areas for 3 GalNAc residues. Only the following results remained significantly different. Both patients with myeloma and HIgAN had p values of less than 0.033 when compared with normal controls for the mean percentage peak area of 5 GalNAc residues. The differences found in the mean

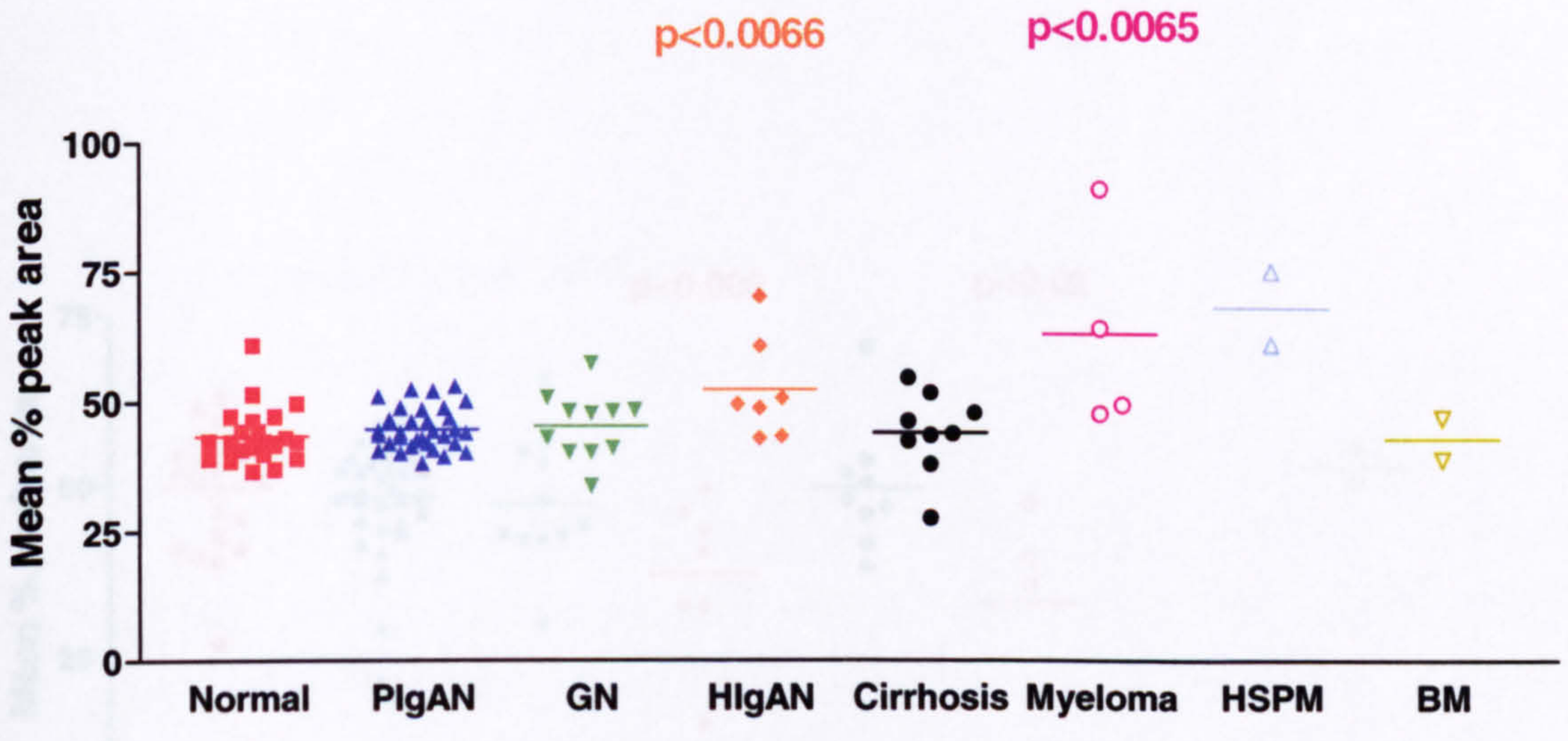



**Figure 5.3** Scatter graph showing the difference in percentage peak area of glycoforms with 4 GalNAc in the study groups. Both patients with hepatic IgAN and myeloma with no renal disease had a greater number of glycoforms with this species present.

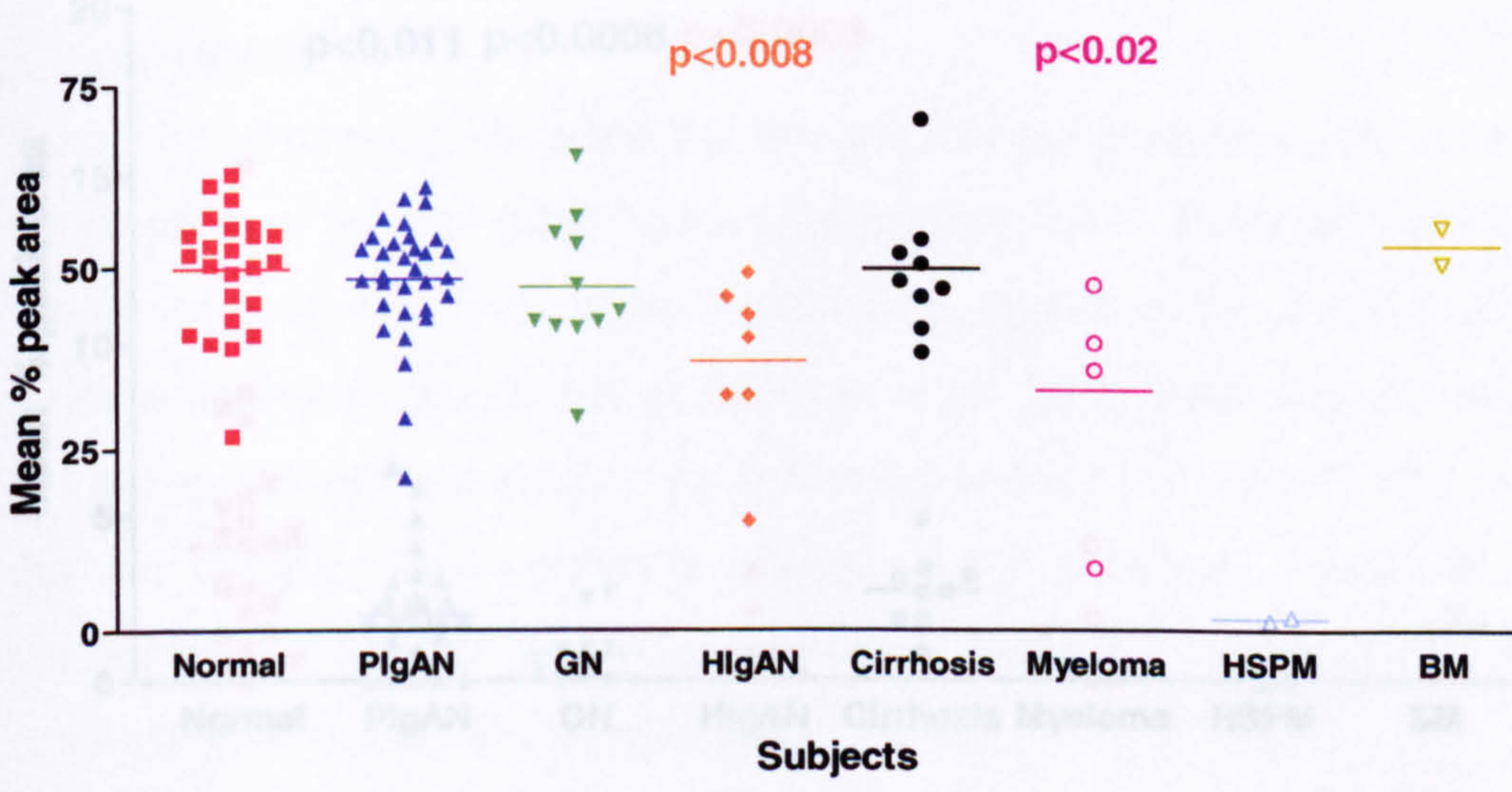



**Figure 5.4** Scatter graph showing the difference in percentage peak area of glycoforms with 5 GalNAc in the study groups. Both patients with hepatic IgAN and myeloma with no renal disease had a lower percentage of glycoforms with this species present. This is a reflection of the increased proportion of the 3 and 4 GalNAc residues present in these patients as shown earlier.

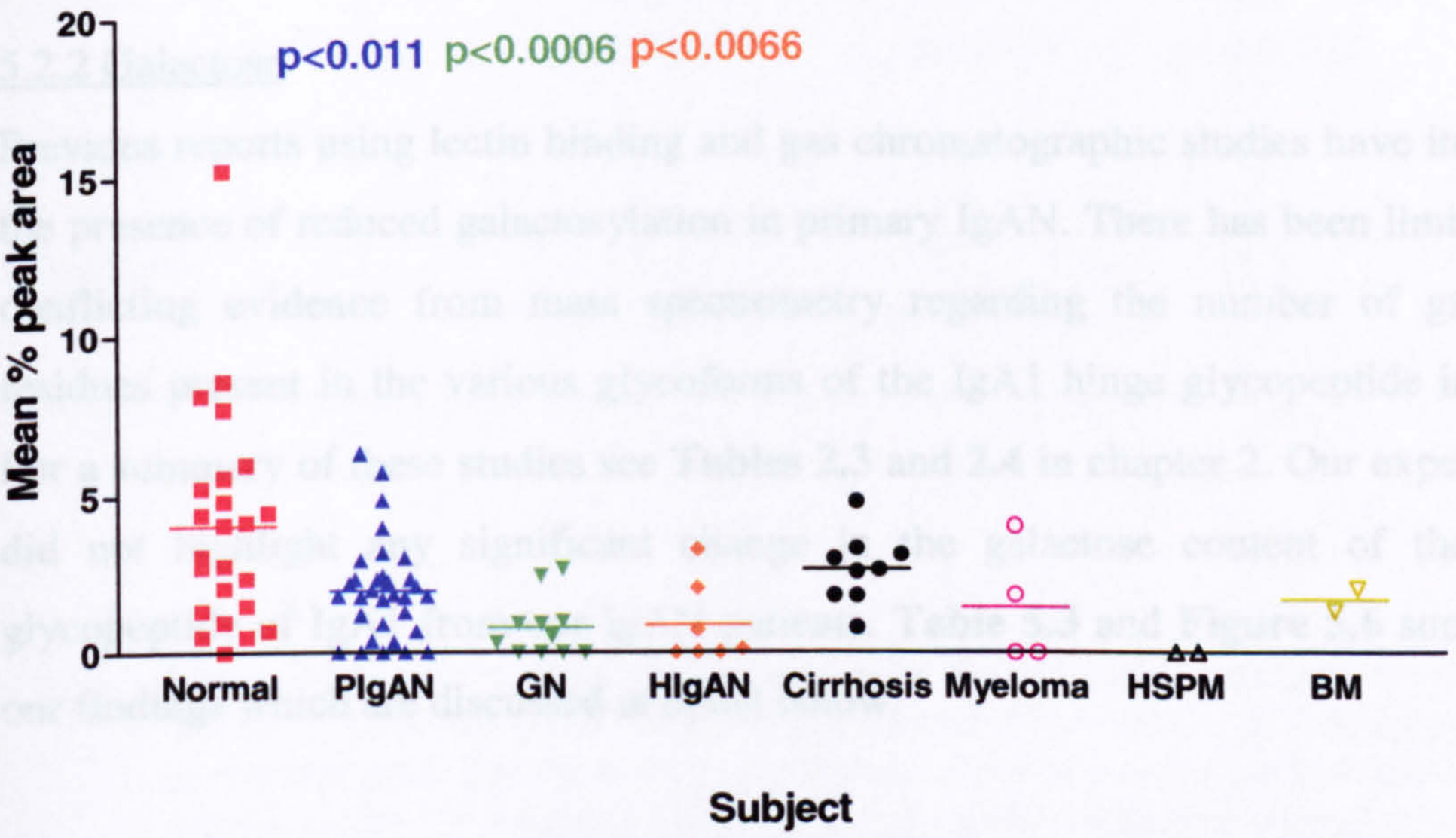



**Figure 5.5** Scatter graph showing the difference in percentage peak area of glycoforms with 6 GalNAc in the study groups. Patients with primary and hepatic IgAN as well as those with non-IgA GN had lower percentage of these glycoforms. This is a reflection of the higher percentage of the glycoforms containing 3 and 4 GalNAc residues.

percentage peak area for 6 GalNAc residues in patients with PIgAN, HIgAN, and other GN also remained significantly different from normal controls with p values of less than 0.05, 0.033, and 0.003 respectively. It is interesting to note that the lowest number of GalNAc residues was found to occur in patients with non-IgA glomerulonephritis. A summary of these results is presented in **Table 5.2.**

This is the first report of a diminished number of GalNAc substitutions of the IgA1 HGP in patients with IgA nephropathies, myeloma, and other glomerulonephritides. Interestingly cirrhosis per se does not appear to be associated with changes in the number of GalNAc substitutions but renal diseases of different aetiology do.

### 5.2.2 Galactose

Previous reports using lectin binding and gas chromatographic studies have indicated the presence of reduced galactosylation in primary IgAN. There has been limited and conflicting evidence from mass spectrometry regarding the number of galactose residues present in the various glycoforms of the IgA1 hinge glycopeptide in IgAN For a summary of these studies see **Tables 2.3** and **2.4** in chapter 2. Our experiments did not highlight any significant change in the galactose content of the hinge glycopeptide of IgA1 from our IgAN patients. **Table 5.3** and **Figure 5.6** summarise our findings which are discussed in detail below.

Having examined the galactose content of the different groups by MALDI, we found that all the groups had peaks corresponding to at least one galactose residue being present and there were no glycoforms containing zero galactose residues (**figure 5.6**). The mean galactose residue per IgA1 molecule in normal controls was 3.6 (**Table 5.1**). The mode for galactosylation was the presence of 4 galactose residues in all groups. We found no statistically significant difference between the patients and controls in the proportion of glycoforms with 1 or 2 galactose residues. However, we did find that a greater proportion of PIgAN patients showed evidence of under-galactosylation with an increase in the mean percentage peak area of species containing only 3 Gal and a proportional reduction in the number of 4 Gal residues both of which attained a slight statistical significance (**figures 5.7 and 5.8**). The mean percentage peak area for 3 Gal and 4 Gal in normal controls was 31.0 ± 9.4 and 62.5 ±11.6 respectively.

| GalNAc | 3 | 4 | 5 | 6 | Content |
|---|---|---|---|---|---|
| **Normal** | 1.9 | 43.4 | 49.8 | 4.1 | - |
| **IgAN** | 3.7 (p<0.04) | 44.7 | 48.5 | 2.0 (p<0.011)‡ | ↓ |
| **GN** | 4.1 | 45.3 | 47.3 | 0.8 (p<0.0006)‡ | (↓) |
| **HIgAN** | 7.1 (p<0.02) | 52.3 (p<0.0066)‡ | 37 (p<0.008)‡ | 0.9 (p<0.007)‡ | ↓↓ |
| **Cirrhosis** | 2.8 | 44.1 | 50.4 | 2.7 | ↔ |
| **Myeloma** | 2.2 | 63.2 (p<0.0065)‡ | 33.1 (p<0.02) | 1.5 | Specific |

**Table 5.2** Summary of the significant results of the GalNAc composition of the IgA1 HGP compared with normal controls. The values are the mean percentage peak area for each group with p values of less than 0.05 deemed significant by the Mann Whitney U test. ‡ denotes statistical significance after applying the Bonferroni correction.

| Gal | 0 | 1 | 2 | 3 | 4 | 5 | Gal content |
|---|---|---|---|---|---|---|---|
| **Normal** | 0 | 0.01 | 3.8 | 31.0 | 62.4 | 1.8 | - |
| **PIgAN** | 0 | 0.6 | 6.4 | 37.8 ($p<0.013$) | 53.3 ($p<0.02$) | 0.8 | ↓ |
| **GN** | 0 | 0.5 | 6 | 33.9 | 55.6 | 1.5 | ↔ |
| **HIgAN** | 0 | 0.4 | 1.7 | 36.2 | 56.1 | 2.9 | ↔ |
| **Cirrhosis** | 0 | 0.2 | 3.1 | 30.2 | 61.9 | 3.9 ($p<0.02$) | ↔ |
| **Myeloma** | 0 | 0 | 0 | 7.2 ($p<0.003$)‡ | 82.3 ($p<0.01$)‡ | 9.8 ($p<0.01$) | ↑ |

**Table 5.3** Summary of the significant results of the Galactose composition of the IgA1 HGP. The values are the mean percentage peak area for number of galactose residues for each group. p values of less than 0.05 were deemed significant by the MWU test when compared with normal controls. The‡ denotes statistical significance after applying the Bonferroni correction.

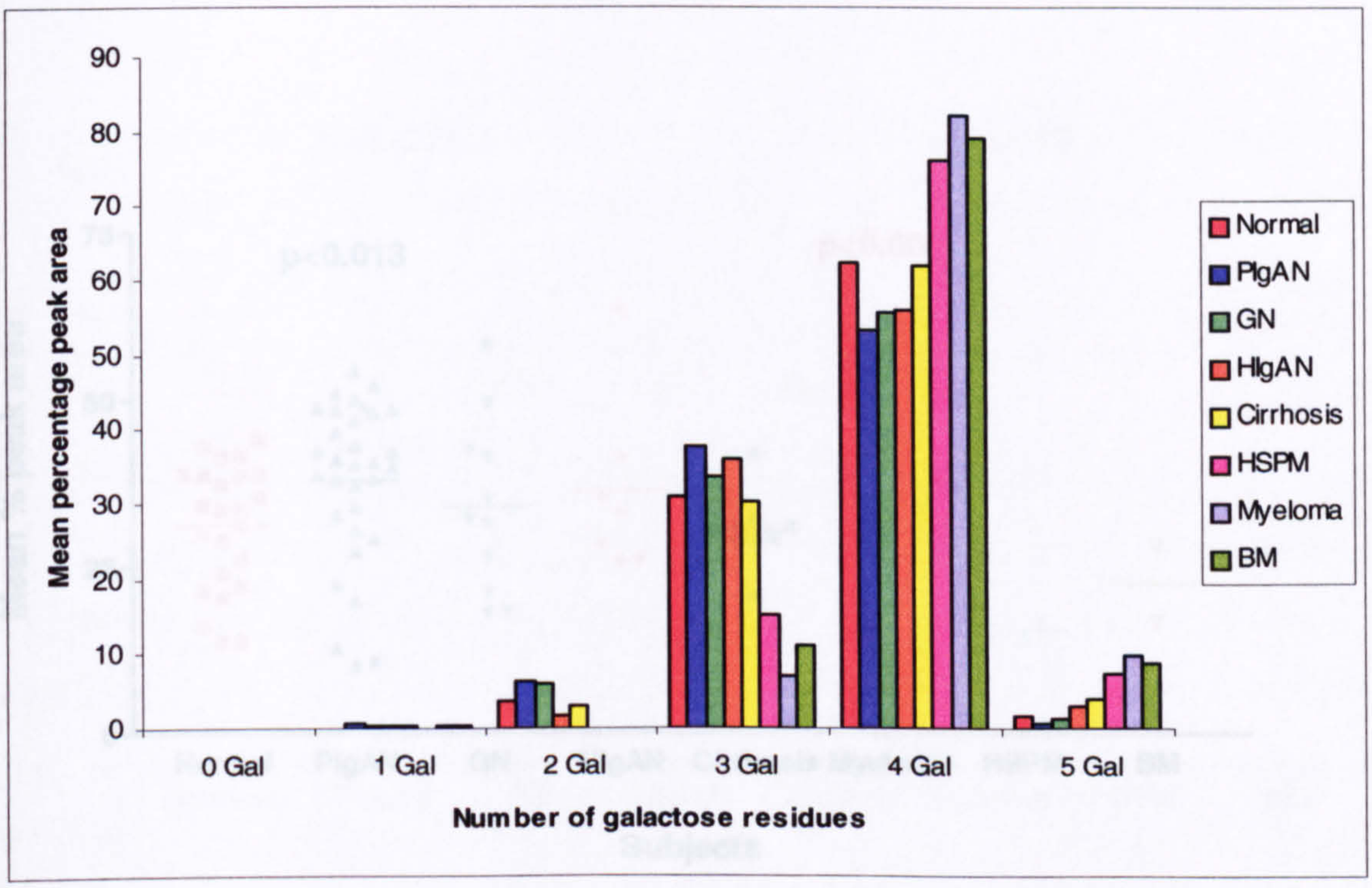



**Figure 5.6** Number of galactose residues in patient and control groups. There were no glycoforms containing no galactose residues in any of the groups. No differences were observed in those containing 1 galactose residues.

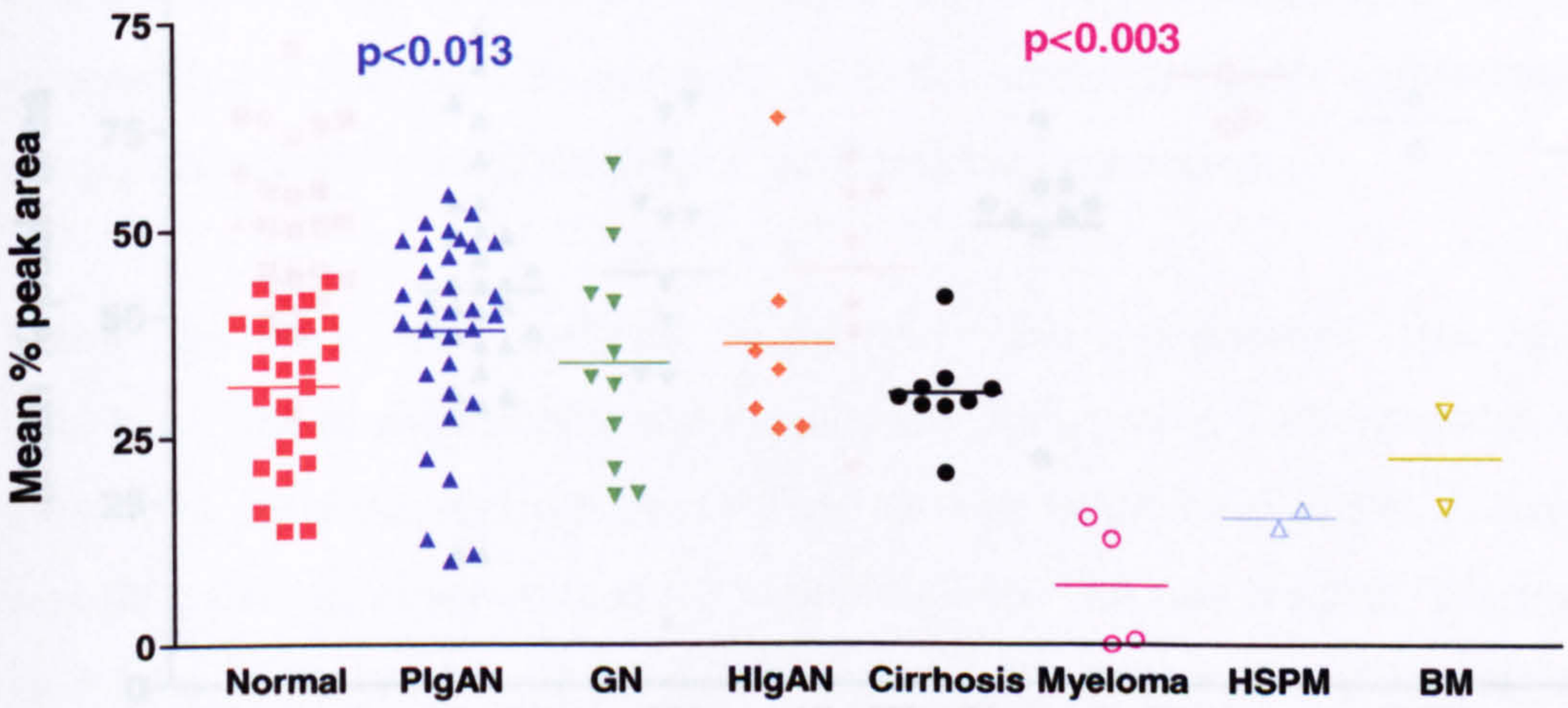



**Figure 5.7** Graph showing the mean percentage peak area of glycoforms containing 3 Galactose residues in the different patient and control groups. Patients with PIgAN showed a statistically significant increase in the proportion of glycoforms containing 3 Gal substitution when compared with normal. Those with myeloma had a very low proportion of glycoforms with 3 Gal.

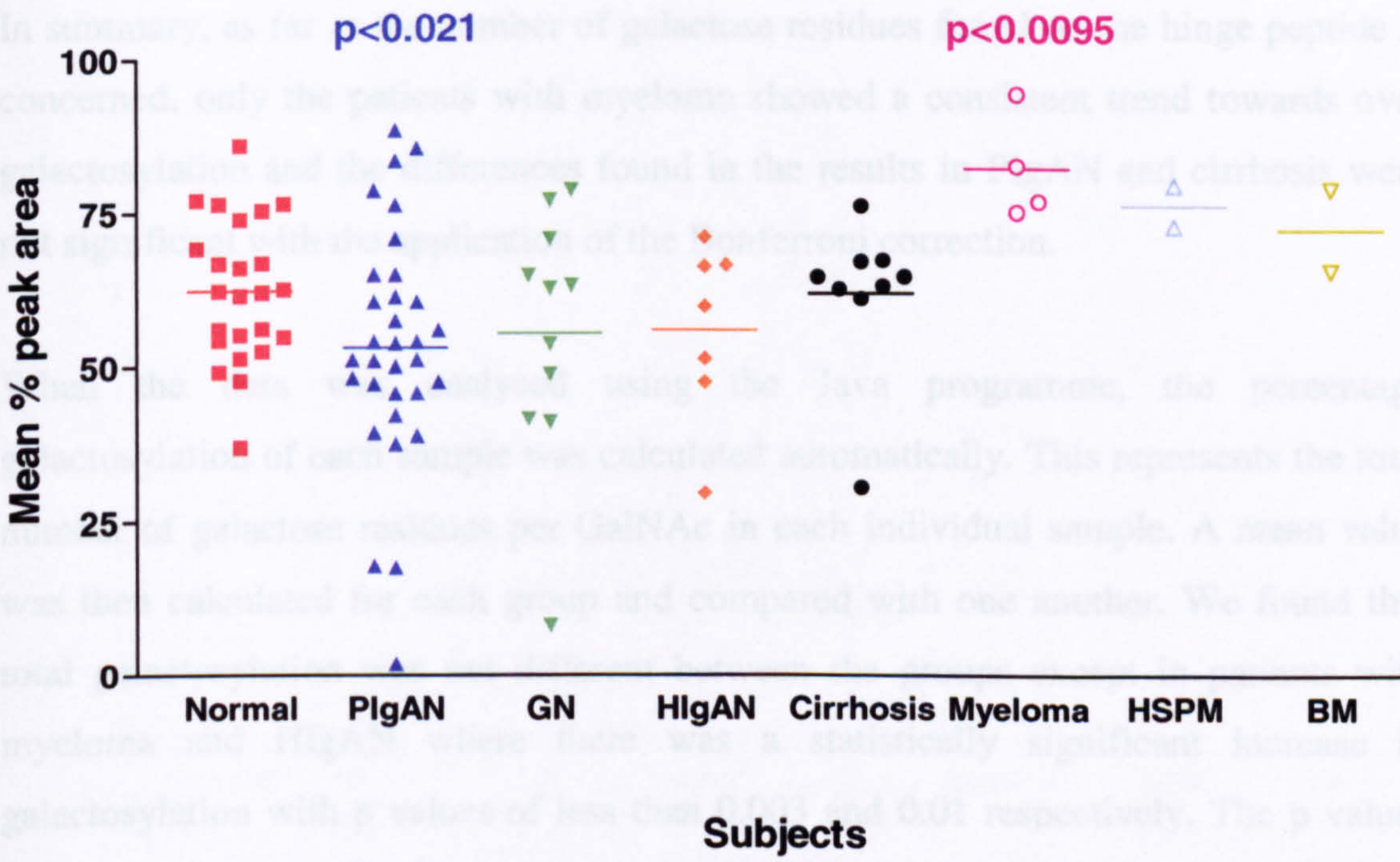



**Figure 5.8** Graph demonstrating the presence of a lower percentage of 4 Gal species in patients with PIgAN as compared with normal. The reverse is seen in patients with myeloma were the proportion of 4 Gal substitutions are increased. This is a reflection of the decreased percentage of the 3 Gal substitutions in this group.

In PIgAN they were 37.8 ±12.1 ($p<0.013$) for 3 Gal and 53.3 +/-19.1 ($p<0.021$) for 4 Gal respectively. The reverse was true in patients with myeloma where the percentage of species containing 4 and 5 Gal was significantly increased whereas there were fewer species containing 3 Gal (**figures 5.7, 5.8, and 5.9**). Patients with cirrhosis also had a slightly higher proportion of species containing 5 galactose residues ($p<0.022$) compared with normal controls suggesting some degree of over-galactosylation. The trend in IgA1 obtained from breast milk is towards higher number of galactose substitutions.

In summary, as far as the number of galactose residues found on the hinge peptide is concerned, only the patients with myeloma showed a consistent trend towards over galactosylation and the differences found in the results in PIgAN and cirrhosis were not significant with the application of the Bonferroni correction.

When the data was analysed using the Java programme, the percentage galactosylation of each sample was calculated automatically. This represents the total number of galactose residues per GalNAc in each individual sample. A mean value was then calculated for each group and compared with one another. We found that total galactosylation was not different between the groups except in patients with myeloma and HIgAN where there was a statistically significant increase in galactosylation with p values of less than 0.003 and 0.01 respectively. The p values after applying the Bonferroni correction remained significant at 0.015 and 0.05 respectively. This data is summarised in **figure 5.10**. Our results therefore do not support the previous findings inferred from lectin binding and chromatographic studies where significant changes in the galactose content of patients with IgA nephropathy have been reported.

### 5.2.3 Sialic Acid

The most salient changes in glycosylation were found in the studies considering the abundance of sialic acid. Glycoforms were found with between nought to 4 NeuNAc residues and the mode in the normal individuals appeared to be glycoforms containing 2 or 3 sialic acids (**figure 5.11**). The mean value calculated for normal controls was 2.42 and both patients with primary and hepatic IgAN as well as those with cirrhosis

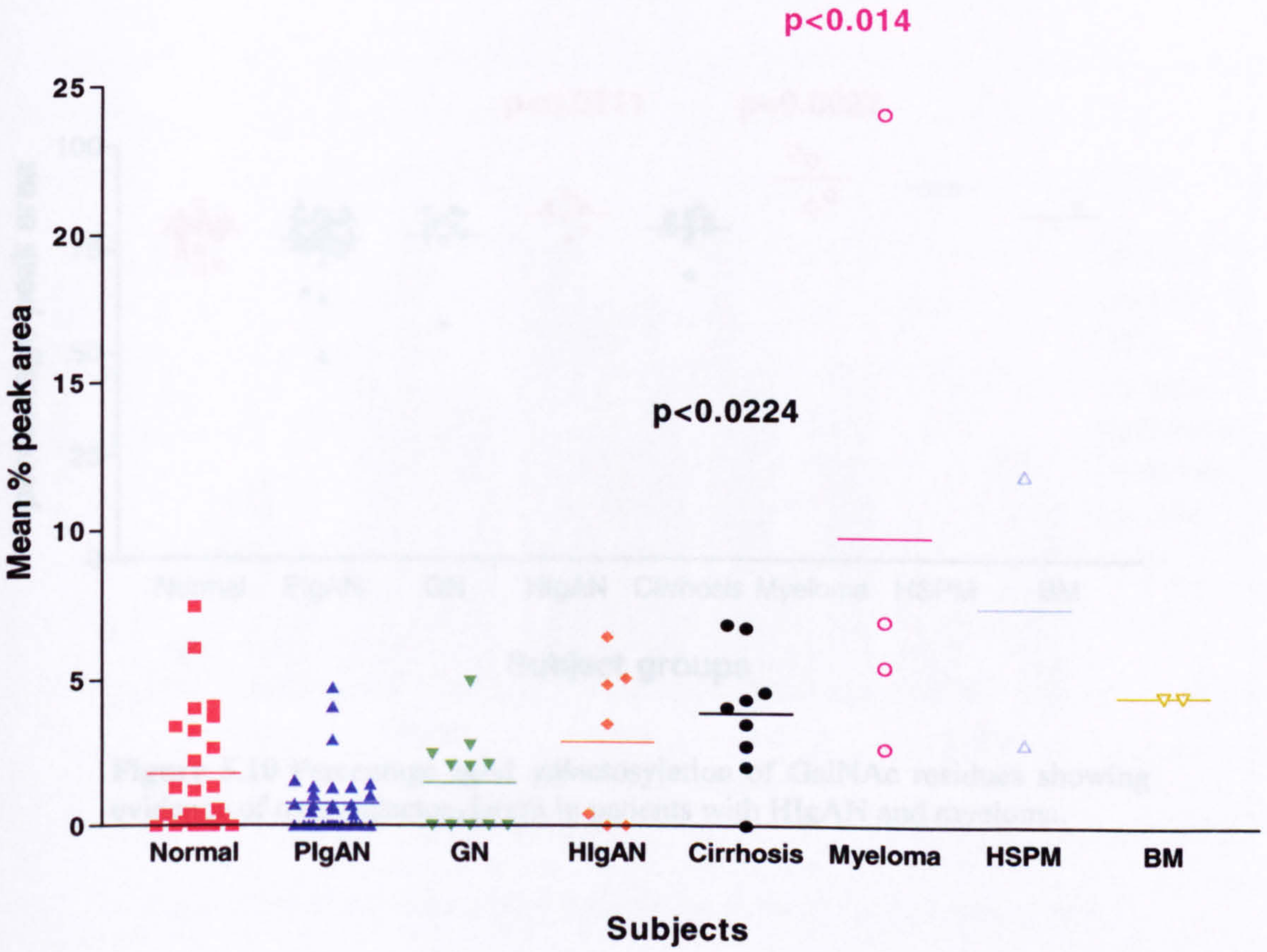



**Figure 5.9** Scatter graph showing 5 Gal substitutions in the patient and control groups. Both patients with myeloma and cirrhosis with no renal disease had higher proportion of the 5 Gal species.

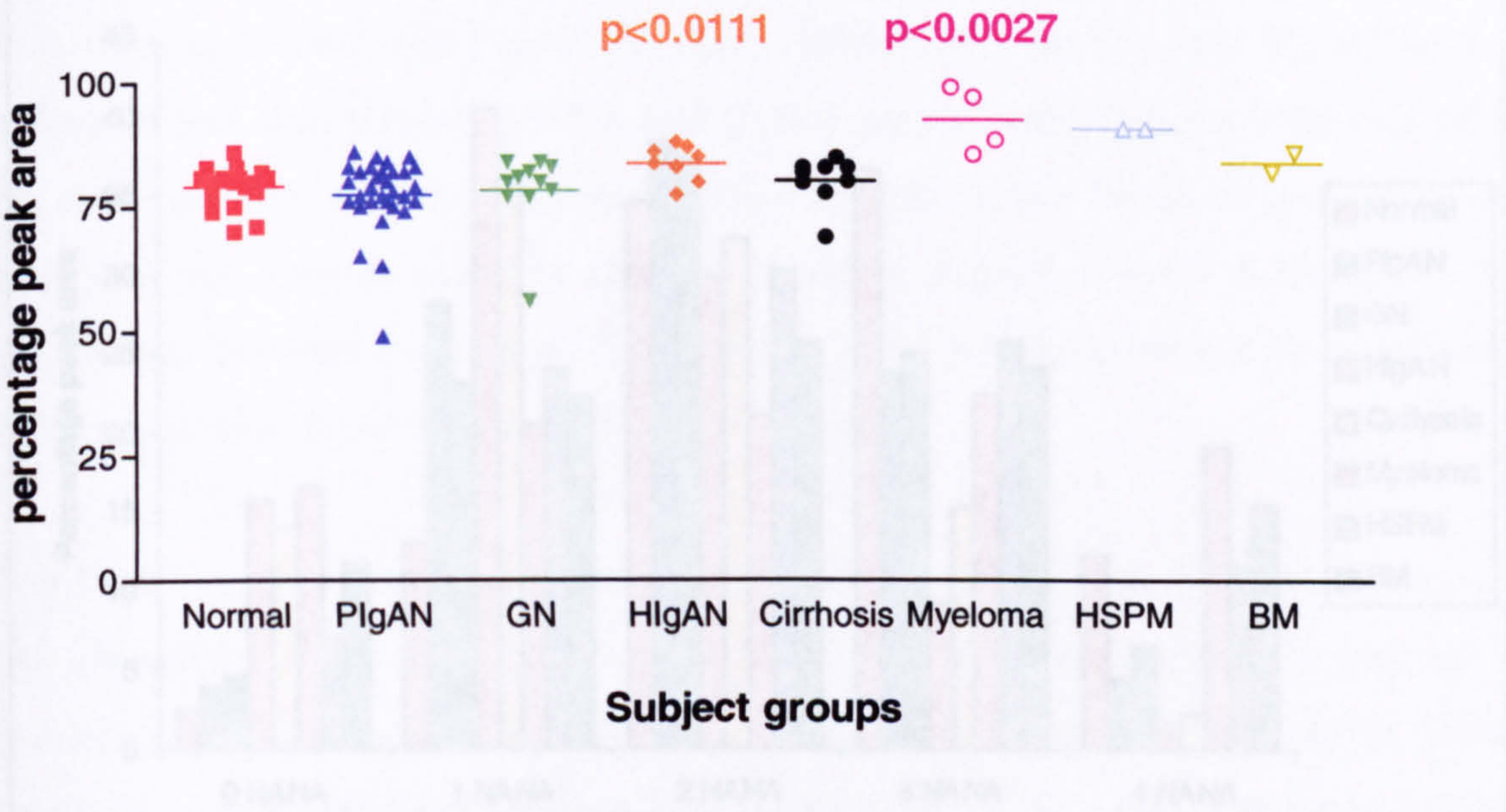



**Figure 5.10** Percentage IgA1 galactosylation of GalNAc residues showing evidence of over-galactosylation in patients with HIgAN and myeloma.

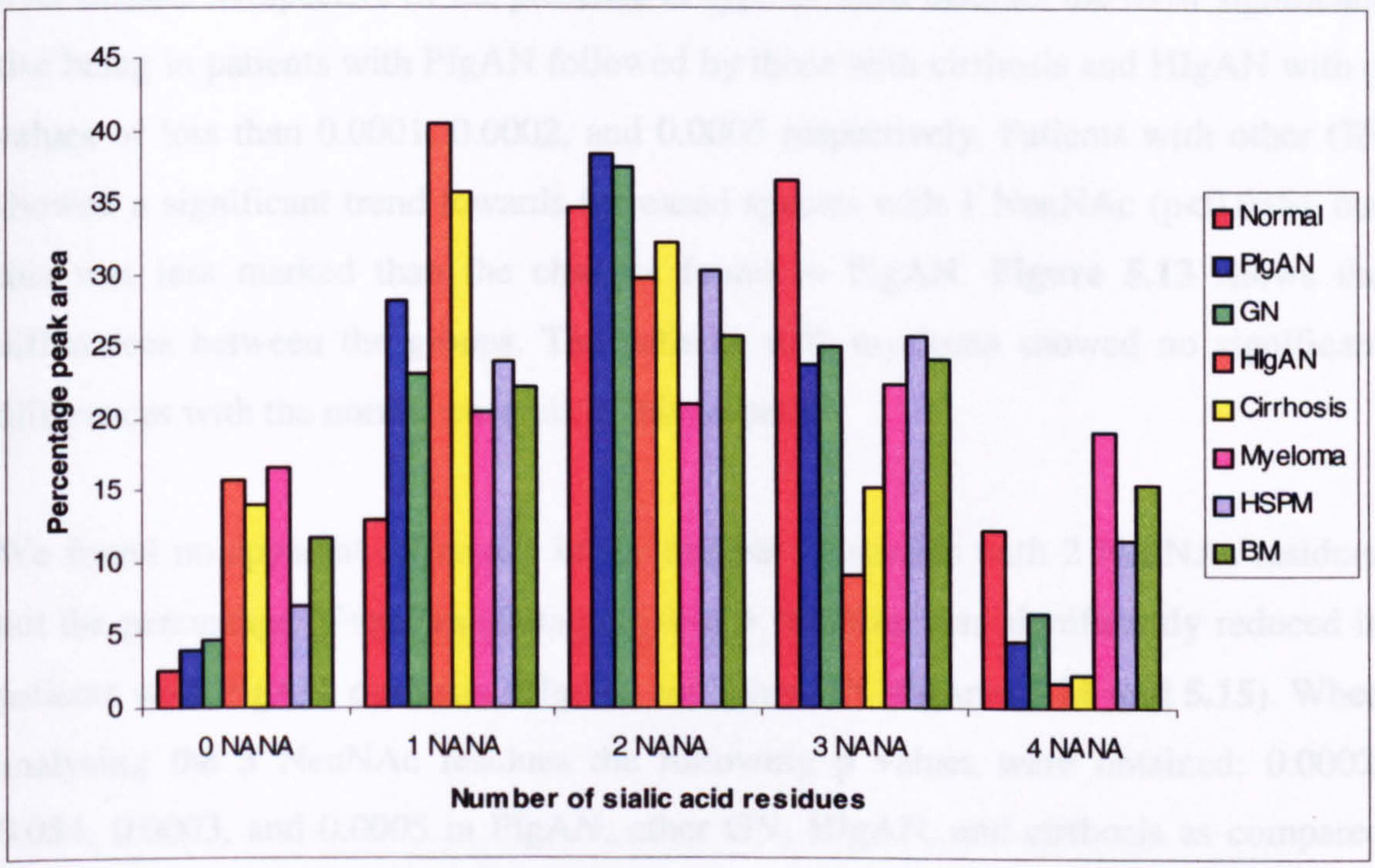



**Figure 5.11** Number of sialic acid residues in the different study groups

without renal disease had significantly reduced sialic acid numbers at 1.94, 1.35, and 1.55 respectively **(Table 5.1)**.

In normal controls the 0 NeuNAc species comprise only 2.6% of the total peak area and by comparison they are significantly elevated only in patients with HIgAN (mean percentage peak area of 15.7 ±14.2, $p<0.016$) **figure 5.12.** However the proportion of glycoforms with 1 NeuNAc was significantly elevated in all patients with renal and liver disease irrespective of the presence or type of renal disease, the most significant rise being in patients with PIgAN followed by those with cirrhosis and HIgAN with p values of less than 0.0001, 0.0002, and 0.0005 respectively. Patients with other GN showed a significant trend towards increased species with 1 NeuNAc ($p<0.035$), but this was less marked than the changes found in PIgAN. **Figure 5.13** shows the differences between the groups. The patients with myeloma showed no significant differences with the normal controls in this respect.

We found no apparent difference in the number of species with 2 NeuNAc residues but the percentage of species containing 3 or 4 NeuNAc was significantly reduced in patients with PIgAN, cirrhosis, HIgAN, and other GN **(figures 5.14 and 5.15)**. When analysing the 3 NeuNAc residues the following p values were obtained: 0.0002, 0.034, 0.0003, and 0.0005 in PIgAN, other GN, HIgAN, and cirrhosis as compared with normal controls respectively. There was no significant change found in patients with myeloma. Likewise, in the analysis of the 4 NeuNAc residues the results were significant on a Mann-Whitney U test with p values of $< 0.001$, 0.03, 0.001, and 0.0001 in the PIgAN, GN, HIgAN, and cirrhotic group respectively as compared with normal controls. After the application of the Bonferroni, the differences between the GN group and normal controls were no longer significant.

When the data were analysed by the Java programme for percentage sialylation of the GalNAc residues, the changes found above were reflected in the analyses **(Figure 5.16)**. Patients with PIgAN and HIgAN were significantly under-sialylated as compared with normal controls ($p<0.0042$ and 0.001 respectively). This was also true of patients with cirrhosis ($p<0.002$) but not those with GN ($p< 0.165$). These changes were maintained when the results were subjected to the Bonferroni test. The sialylation changes and their statistical significance are summarised in **Table 5.4.**

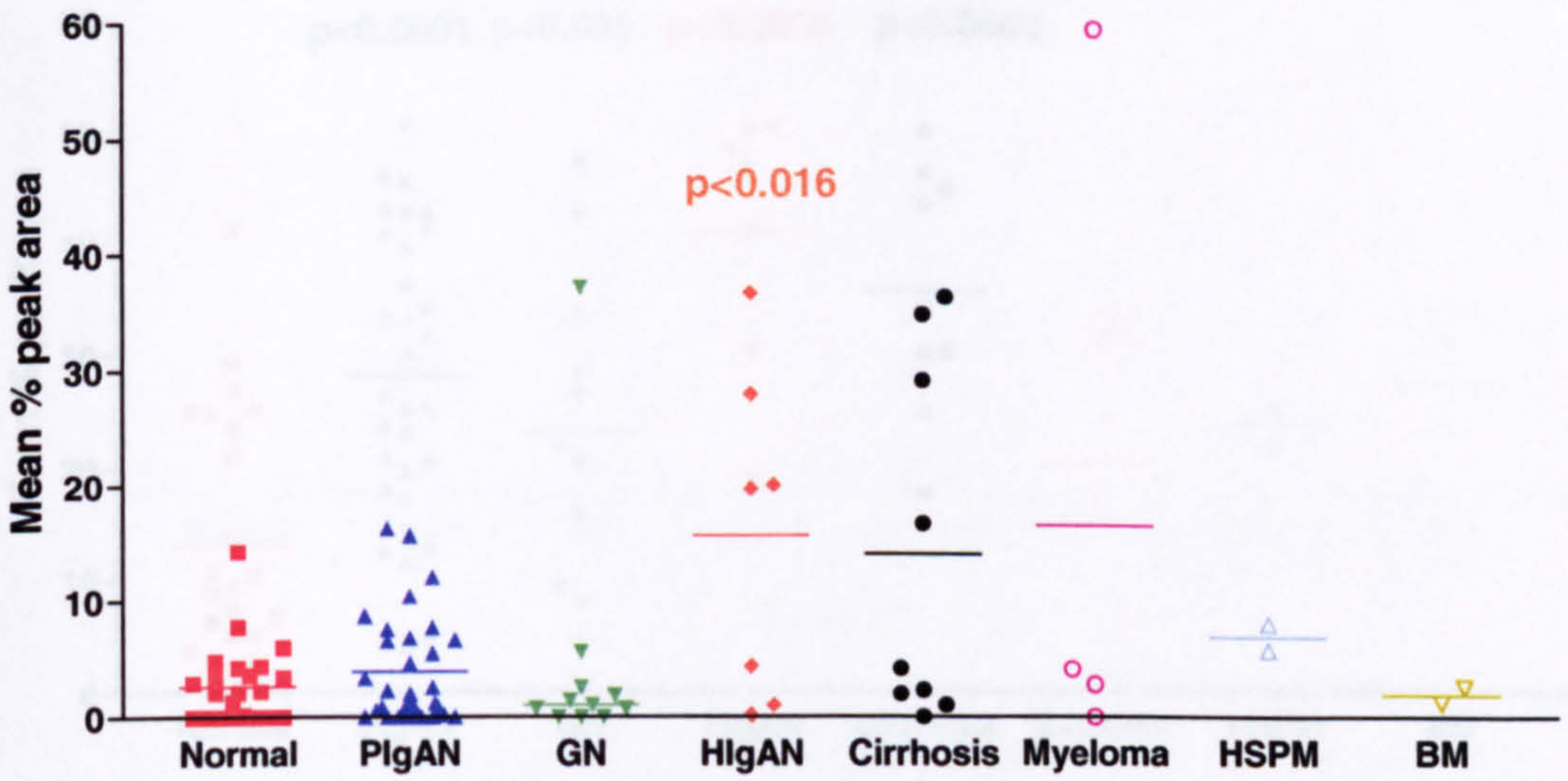



**Figure 5.12** Scatter graph comparing the percentage peak area of glycoforms containing zero sialic acid residues. Even though both patients with HIgAN and cirrhosis appeared to have elevated levels of 0 NeuNAc, only in patients with hepatic IgAN did this achieve statistical significance as compared with normal.

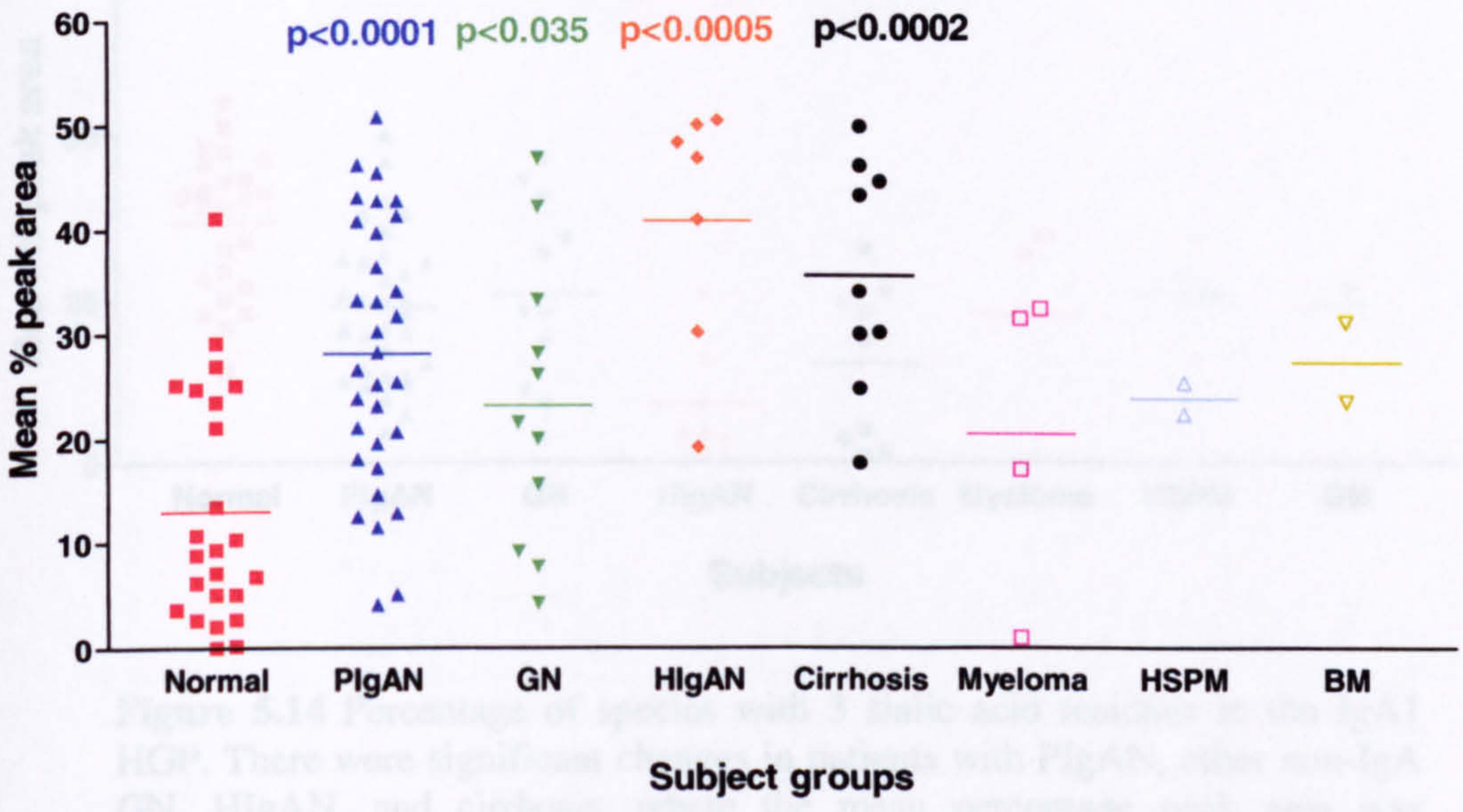



**Figure 5.13** Scatter graph showing the differences between the groups in percentage peak area of glycoforms with 1 sialic acid residue. The levels were statistically elevated in patients with PIgAN, other GN, HIgAN, and cirrhosis.

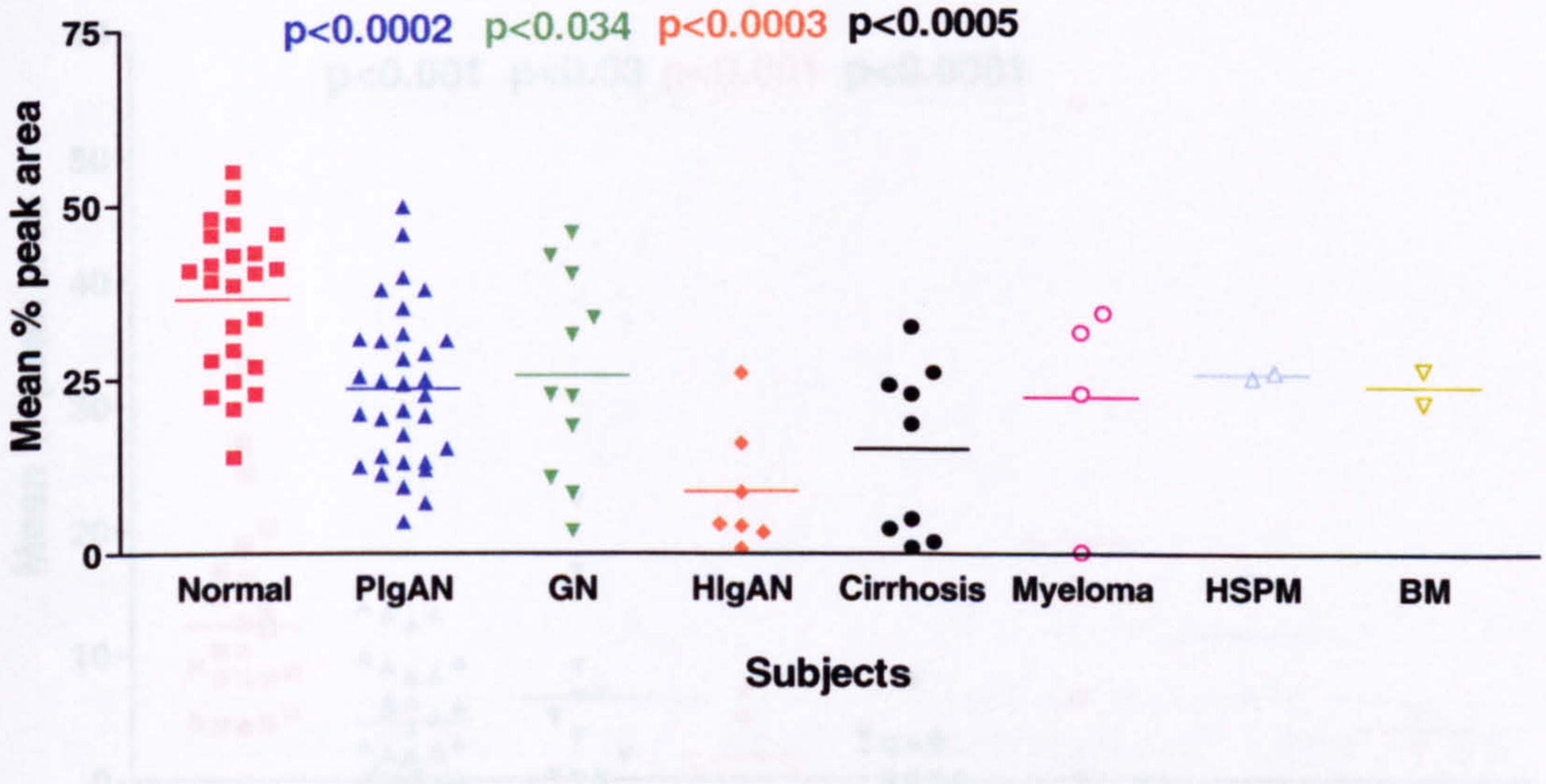



**Figure 5.14** Percentage of species with 3 sialic acid residues in the IgA1 HGP. There were significant changes in patients with PIgAN, other non-IgA GN, HIgAN, and cirrhosis, where the mean percentage peak area was reduced compared with normal controls. No statistically significant difference was apparent in the patients with myeloma or in the composition of breast milk IgA1.

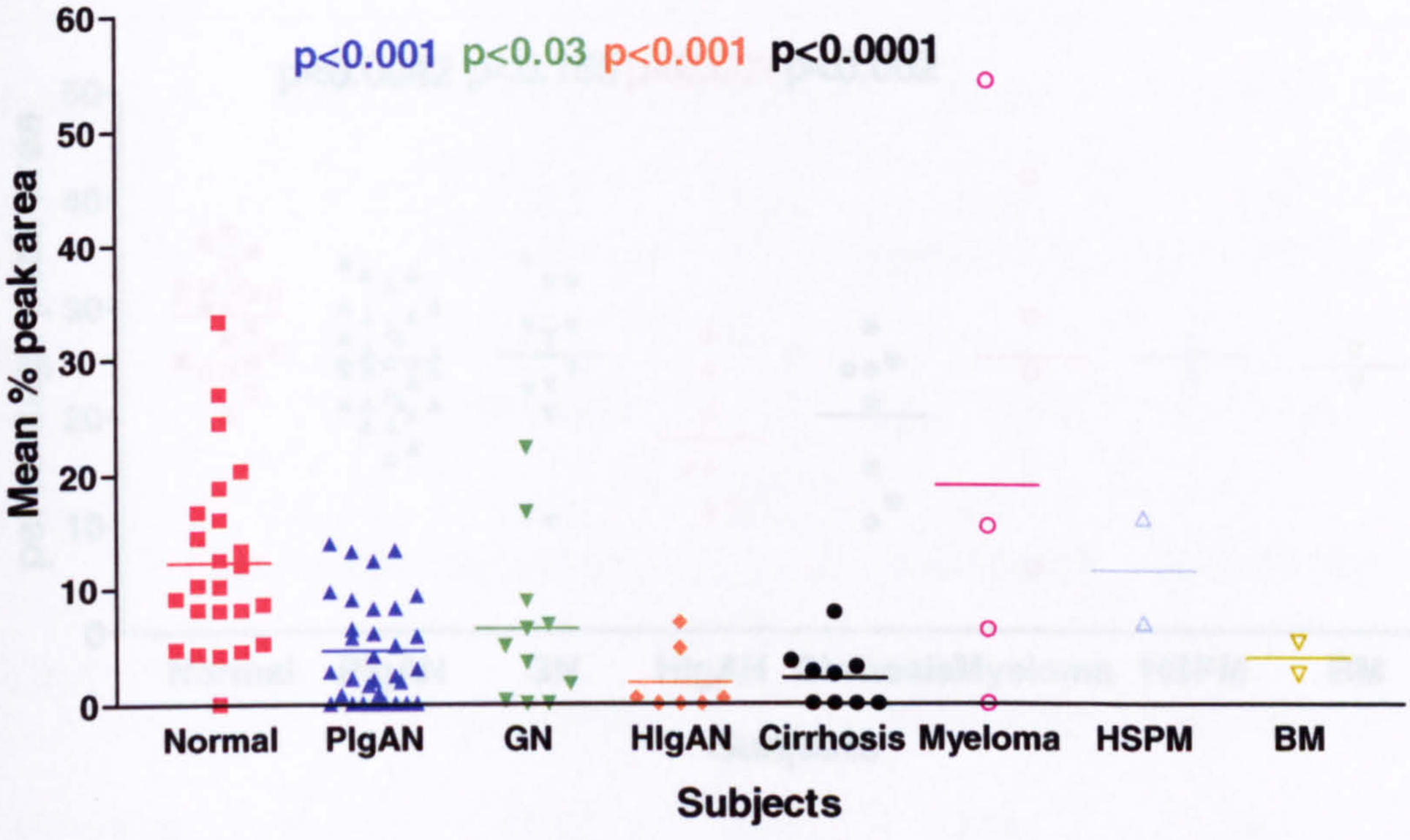



**Figure 5.15** Percentage of species with 4 sialic acid residues in the IgA1 HGP. There were significant changes in patients with PIgAN, other non-IgA GN, HIgAN, and cirrhosis, where the mean percentage peak area was reduced compared with normal controls. No statistically significant difference was apparent in the patients with myeloma or in the composition of breast milk IgA1.

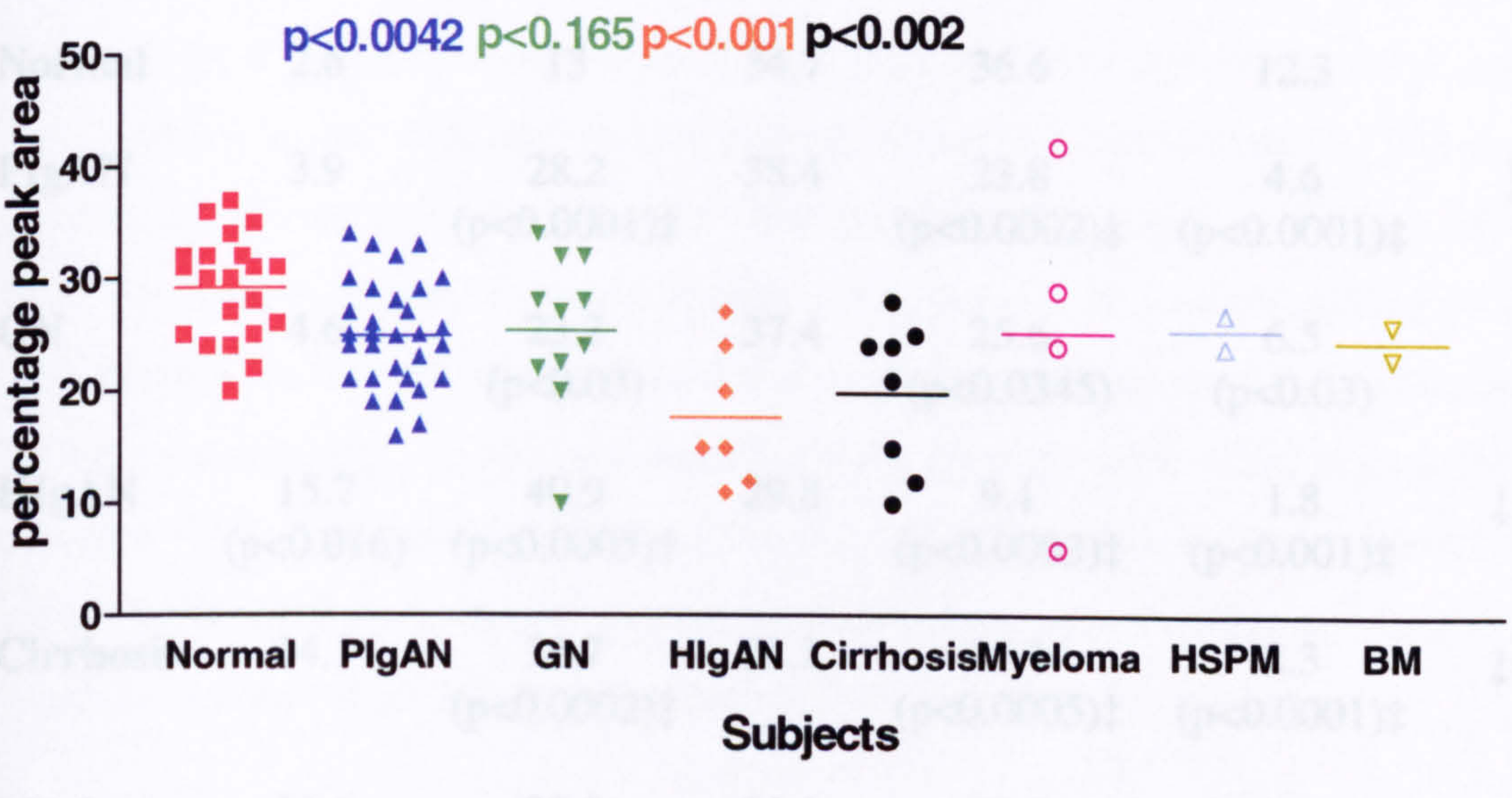



**Figure 5.16** Percentage sialylation of the IgA1 hinge glycopeptide in the patient and control groups. Patients with PIgAN, GN, HIgAN, and cirrhosis all showed evidence of under-sialylation.

| NANA | 0 | 1 | 2 | 3 | 4 | NANA content |
|---|---|---|---|---|---|---|
| **Normal** | 2.6 | 13 | 34.7 | 36.6 | 12.3 | - |
| **PIgAN** | 3.9 | 28.2 (p<0.0001)‡ | 38.4 | 23.8 (p<0.0002)‡ | 4.6 (p<0.0001)‡ | ↓↓ |
| **GN** | 4.6 | 23.2 (p<0.03) | 37.4 | 25.6 (p<0.0345) | 6.5 (p<0.03) | ↓ |
| **HIgAN** | 15.7 (p<0.016) | 40.9 (p<0.0005)‡ | 29.8 | 9.1 (p<0.0003)‡ | 1.8 (p<0.001)‡ | ↓↓↓ |
| **Cirrhosis** | 14.1 | 35.7 (p<0.0002)‡ | 32.2 | 15.1 (p<0.0005)‡ | 2.3 (p<0.0001)‡ | ↓↓↓ |
| **Myeloma** | 16.6 | 20.5 | 21.1 | 22.5 | 19.1 | ↔ |

**Table 5.4** Summary of the significant results of the sialic acid composition of the IgA1 HGP. The values are the mean percentage peak area for each group with p values of less than 0.05 deemed significant by the MWU test with respect to normal controls. The ‡ denotes statistical significance after applying the Bonferroni correction.

In summary, in our MALDI analyses undersialylation appears to be a prominent feature of the IgA1 structure in patients with PIgAN, HIgAN, and cirrhosis.

## 5.3 Glycan ranking in order of abundance

The various glycoforms identified by mass spectrometry were tabulated in rank order as seen in **tables 5.5 and 5.6.**

### 5.3.1 Rank order

The ranking of the glycoforms shed further light on the composition of the various IgA1 species in the different subject groups. In normal individuals the commonest glycoforms appear to be the more abundantly glycosylated species such as 443 and 543. The least abundant or absent species were those with lower numbers of sialic acid residues. In the top ten glycoforms, although the ranking varied to a minor degree between patients with PIgAN and normal, similar changes were found in patients with non-IgA GNs. The ranking between the PIgAN group and its renal control were virtually identical in the major species. However the most significant changes were found in the least abundant species which will be discussed further below.

There were obvious differences in terms of the ranking of species between the normal controls and patients with liver disease. Unlike in the normal individual, both HIgAN and cirrhosis patients had higher ranking glycoforms with only one, two or even no sialic acid residues. In patients with myeloma with and without renal disease, the striking finding was the simplification of the spectra with fewer glycoforms being present. In the myeloma group the highest ranking glycoforms were those containing 4 GalNAc and 4 Gal substitutions with varying degrees of sialylation. In contrast those with myeloma leading to HSP showed a pattern with a whole variety of glycoforms not too dissimilar to the normal.

### 5.3.2 Additions

In each of the patient groups we were able to identify a number of glycoforms that were specific to that group. These are shown in **Table 5.7.** The glycoforms, in order of abundance, that were specific to the PIgAN group and not the normal and renal (GN) control groups were: 420, 630, 320, 611, 321, 400. These were only present in less than one percent of the total peak area for each group. The 331, 411, and 511

| Rank | PIGAN | GN | HIGAN | C | N | HSPM | M | BM |
|---|---|---|---|---|---|---|---|---|
| 1 | 542 | 442 | 441 | 541 | 443 | 442 | 444 | 542 |
| 2 | 442 | 542 | 442 | 542 | 543 | 443 | 443 | 442 |
| 3 | 443 | 443 | 431 | 442 | 542 | 441 | 442 | 541 |
| 4 | 543 | 543 | 541 | 441 | 442 | 444 | 441 | 443 |
| 5 | 532 | 532 | 542 | 443 | 532 | 541 | 440 | 543 |
| 6 | 541 | 432 | 432 | 543 | 444 | 542 | 541 | 441 |
| 7 | 432 | 541 | 443 | 531 | 432 | 440 | 542 | 532 |
| 8 | 431 | 431 | 531 | 431 | 533 | 543 | 543 | 432 |
| 9 | 441 | 441 | 540 | 532 | 544 | 532 | 550 | 531 |
| 10 | 531 | 531 | 440 | 432 | 541 | 533 | 540 | 431 |
| 11 | 433 | 444 | 532 | 540 | 433 | 552 | 551 | 332 |
| 12 | 533 | 433 | 430 | 440 | 431 | 551 | 553 | 433 |
| 13 | 444 | 533 | 332 | 530 | 441 | 433 | 544 | 544 |
| 14 | 544 | 544 | 543 | 430 | 531 | 432 | 552 | 444 |
| 15 | 521 | 332 | 530 | 551 | 620 | 431 |  | 551 |
| 16 | 332 | 521 | 331 | 433 |  | 544 |  | 533 |
| 17 | 421 | 333 | 550 | 332 |  | 531 |  | 552 |
| 18 | 540 | 520 | 330 | 533 |  | 553 |  |  |
| 19 | 422 | 540 |  | 444 |  | 540 |  |  |
| 20 | 333 | 530 |  | 552 |  |  |  |  |
| 21 | 530 |  |  |  |  |  |  |  |

**Table 5.5** Presentation of glycoforms in rank order which consist of greater than one percent of the mean total peak area for each group. The top ten glycoforms (separated with the horizontal red line) from normal controls have been highlighted in blue in the normal subjects as well as the other patient and control groups for ease of comparison between the groups. It is clear that the highest-ranking glycoforms in the normal subjects, with a few exceptions, also represent the most abundant forms in the other groups.

| PIGAN | GN | HIGAN | C | N | HSPM | M | BM |
|---|---|---|---|---|---|---|---|
| 522 | 421 | 444 | 544 | 332 | 550 | 332 | 632 |
| 631 | 522 | 551 | 631 | 333 | 430 | 333 | 531 |
| 430 | 422 | 433 | 421 | 632 | 332 | 532 | 540 |
| 632 | 552 | 544 | 331 | 552 | 320 | 432 | 550 |
| 322 | 430 | 333 | 632 | 621 | 321 | 531 | 553 |
| 440 | 322 | 552 | 550 | 633 | 322 | 433 | 440 |
| 551 | 440 | 322 | 521 | 551 | 330 | 642 | 422 |
| 552 | 551 | 533 | 420 | 553 | 331 | 554 | 331 |
| 511 | 331 | 511 | 333 | 422 | 333 | 511 | 521 |
| 331 | 553 | 631 | 520 | 530 | 400 | 533 | 522 |
| 621 | 632 | 630 | 422 | 522 | 411 | 431 | 320 |
| 620 | 511 | 420 | 553 | 521 | 420 | 631 | 321 |
| 411 | 633 | 521 | 630 | 540 | 421 | 632 | 322 |
| 520 | 620 | 553 | 620 | 421 | 422 | 641 | 330 |
| 633 | 411 | 641 | 621 | 631 | 510 | 530 | 333 |
| 420 | 554 | 321 | 321 | 322 | 511 | 331 | 400 |
| 630 | 631 | 421 | 641 | 520 | 520 | 320 | 411 |
| 510 | 642 | 520 | 633 | 550 | 521 | 321 | 420 |
| 320 | 652 | 620 | 510 | 642 | 522 | 322 | 421 |
| 611 | 320 | 422 | 642 | 643 | 530 | 330 | 430 |
| 553 | 321 | 522 | 650 | 554 | 554 | 400 | 510 |
| 550 | 330 | 554 | 610 | 440 | 610 | 411 | 511 |
| 321 | 400 | 510 | 522 | 430 | 611 | 420 | 520 |
| 400 | 420 | 610 | 330 | 510 | 620 | 421 | 530 |
| 330 | 510 | 320 | 320 | 652 | 621 | 422 | 554 |
| 554 | 550 | 400 | 322 | 644 | 630 | 430 | 610 |
| 610 | 610 | 411 | 400 | 320 | 631 | 510 | 611 |
| 641 | 611 | 611 | 411 | 321 | 632 | 520 | 620 |
| 642 | 621 | 621 | 511 | 330 | 633 | 521 | 621 |
| 643 | 630 | 632 | 554 | 331 | 641 | 522 | 630 |
| 644 | 641 | 633 | 611 | 400 | 642 | 610 | 633 |
| 650 | 644 | 642 | 643 | 411 | 643 | 611 | 641 |
| 652 | 650 | 643 | 644 | 420 | 644 | 620 | 642 |
|  | 652 | 644 | 652 | 511 | 650 | 621 | 643 |
|  |  | 650 |  | 610 | 652 | 630 | 644 |
|  |  | 652 |  | 611 |  | 633 | 650 |
|  |  |  |  | 630 |  | 643 | 652 |
|  |  |  |  | 641 |  | 644 |  |
|  |  |  |  | 650 |  | 650 |  |
|  |  |  |  |  |  | 652 |  |

**Table 5.6** The glycoforms shown in red are presented in rank order representing those glycans identified with a mean percentage of total peak area of less than one percent. The glycoforms in green are those absent in each group and are not in rank order.

| PIgAN | PIgAN/GN | HIgAN/Cirrhosis | Cirrhosis |
|---|---|---|---|
| 420 | 331 | 331 | 420 |
| 630 | 411 | 330 | 630 |
| 320 | 511 | | 321 |
| 611 | | | 641 |
| 321 | | | 650 |
| 400 | | | 610 |

**Table 5.7** List of glycoforms present specifically in the respective patient groups.

glycoforms were found only in patients with PIgAN, HIgAN, and cirrhosis and not in any of the other groups. In patients with HIgAN, only two glycoforms were found additionally which were not present in the normal controls and these were 331 and 330. In addition to these two glycoforms, patients with cirrhosis also had the following species in their spectra: 420, 630, 321, 641, 650, and 610.

IgA1 HGP purified from breast milk appears to have very similar glycoforms present as compared with the systemic IgA in normal controls, with the addition of the two species 552 and 551. There were no additional glycoforms identified in patients with myeloma.

### 5.3.3 Omissions

In patients with PIgAN there were 5 main glycoforms absent from the spectra as compared with the normal. These were species 554, 652, 642, 643, and 644, the last 3 of which were also not found in patients with other GN. In those with HIgAN, a number of species were missing when compared with normal controls, namely glycoforms 621, 632, 633, 643, 644, and 652. The cirrhotics have also lost the latter 3 glycoforms as well as 322 and 554. Patients with myeloma and HSP myeloma did not contain any 6 GalNAc species and only one species with 3 GalNAc in the latter group. There were many species absent from the relatively simple spectra of these patients which also holds true of the IgA1 from breast milk. These glycoforms are shown in **Table 5.8.**

## 5.4 Correlating glycosylation with lectin binding

Much of the glycosylation work on IgA1 has been done using lectin binding techniques as reviewed in chapter 2. In this study we have demonstrated very good correlation between the lectin binding studies on some of our samples which were subsequently analysed by mass spectrometry. Dr Alice Smith of Leicester University performed lectin binding studies on a number of samples. These same samples then underwent analysis by mass spectrometry in our laboratory. There were 13 patients with PIgAN, 7 normal controls, 4 myeloma patients, and 2 patients with HSP secondary to IgA myeloma. The assays were performed using the three different lectins Vicia villosa (VV), Helix aspersa (HAA), and Peanut agglutinin (PNA) as

| PIgAN | GN | HIgAN | Cirrhosis |
|---|---|---|---|
| 554 | 642 | 621 | 322 |
| 652 | 643 | 632 | 554 |
| 642 | 644 | 633 | 643 |
| 643 | | 643 | 644 |
| 644 | | 644 | 652 |
| | | 652 | |

**Table 5.8** List of glycoforms absent in the respective patient groups compared to those found in the normal controls.

described in the methods and materials chapter. In brief, the assays were performed on serum and not purified IgA1 with the IgA being captured on anti-IgA-coated plates before applying the lectins. Duplicate plates were developed with anti-IgA1 antibody to ensure the wells were saturated with IgA1 and that the differences in lectin binding were not due to different amounts of IgA1 on the plates.

**Table 5.9 and Figure 5.17** contain the results of these studies which show that patients with PIgAN had statistically increased ($p<0.0004$) binding to both HAA and VV with no difference in binding to PNA compared with normals. Patients with myeloma on the other hand showed reduced binding to the VV lectin ($p<0.01$) and increased binding to PNA ($p<0.04$) and no change in the binding pattern to HAA as compared with normal. Both HAA and VV are known to have the strongest affinity for GalNAc amongst lectins. Therefore the higher binding to IgA1 from patients with PIgAN is a result of lower glycosylation. PNA has the strongest affinity for asialylated β-1,3 Gal linked to GalNAc and to a lesser extent to unsubstituted GalNAc. It is extremely inhibited by the presence of sialic acid and therefore binds weakly to native IgA1. The increased binding to myeloma IgA suggests undersialylation of the IgA1 in these patients with no significant changes in patients with primary IgAN being observed, suggesting there are still sufficient number of sialic acid residues present in this group to inhibit the binding to PNA.

We then went on to assess the correlation between binding to these lectins with percentage sialylation and galactosylation by the Spearman r test using 95% confidence intervals to assess statistical significance. **Figure 5.18** shows the correlation between the lectin binding with VV, HAA, and PNA with galactosylation. The results showed significant correlation between the percentage galactosylation and the binding to all 3 lectins. Both HAA and VV showed a significant negative correlation with percentage galactosylation of the purified IgA1 HGP with r values equal to –0.64 ($p<0.0004$) and –0.57 ($p<0.002$) respectively. This is consistent with the finding that the presence of galactose blocks the access of these lectins to the GalNAc residues and lowers their binding, hence the negative correlation. HA and VV also showed negative correlation with sialylation with r values of –0.47 ($p<0.037$) and –0.53 ($p<0.016$) respectively. This negative correlation is because sialic acid will similarly block access to the GalNAc, especially so because of its larger size and the

| Subjects | HA | VV | PNA |
|---|---|---|---|
| PIgAN | 2.02 | 1.65 | 0.32 |
| PIgAN | 1.75 | 1.41 | 0.25 |
| PIgAN | 1.71 | 1.52 | 0.28 |
| PIgAN | 2.48 | 2.23 | 0.24 |
| PIgAN | 1.79 | 1.91 | 0.39 |
| PIgAN | 1.65 | 1.59 | 0.44 |
| PIgAN | 2.45 | 2.30 | 0.23 |
| PIgAN | 1.95 | 1.57 | 0.22 |
| PIgAN | 1.57 | 1.47 | 0.31 |
| PIgAN | 1.73 | 1.42 | 0.14 |
| PIgAN | 1.72 | 1.58 | 0.18 |
| PIgAN | 2.12 | 1.76 | 0.13 |
| PIgAN | 1.56 | 1.48 | 0.26 |
| Normal | 1.17 | 1.16 | 0.26 |
| Normal | 1.38 | 1.19 | 0.18 |
| Normal | 1.38 | 1.20 | 0.18 |
| Normal | 1.04 | 0.86 | 0.21 |
| Normal | 1.29 | 1.23 | 0.27 |
| Normal | 1.36 | 1.18 | 0.25 |
| Normal | 1.33 | 1.21 | 0.17 |
| Myeloma | 1.53 | 0.99 | 0.46 |
| Myeloma | 0.72 | 0.21 | 0.76 |
| Myeloma | 0.84 | 0.69 | 0.22 |
| Myeloma | 0.60 | 0.64 | 0.67 |
| HSPM | 2.04 | 1.53 | 0.50 |
| HSPM | 0.53 | 1.94 | 0.30 |

**Table 5.9** Results from the lectin binding studies, showing the degree of binding of IgA from serum to the lectins Helix aspersa, Vicia villosa, and Peanut agglutinin in patients with primary IgAN, normal controls, myeloma and HSP myeloma. The data shown are the absorbance values for the ELISA-based lectin binding studies.

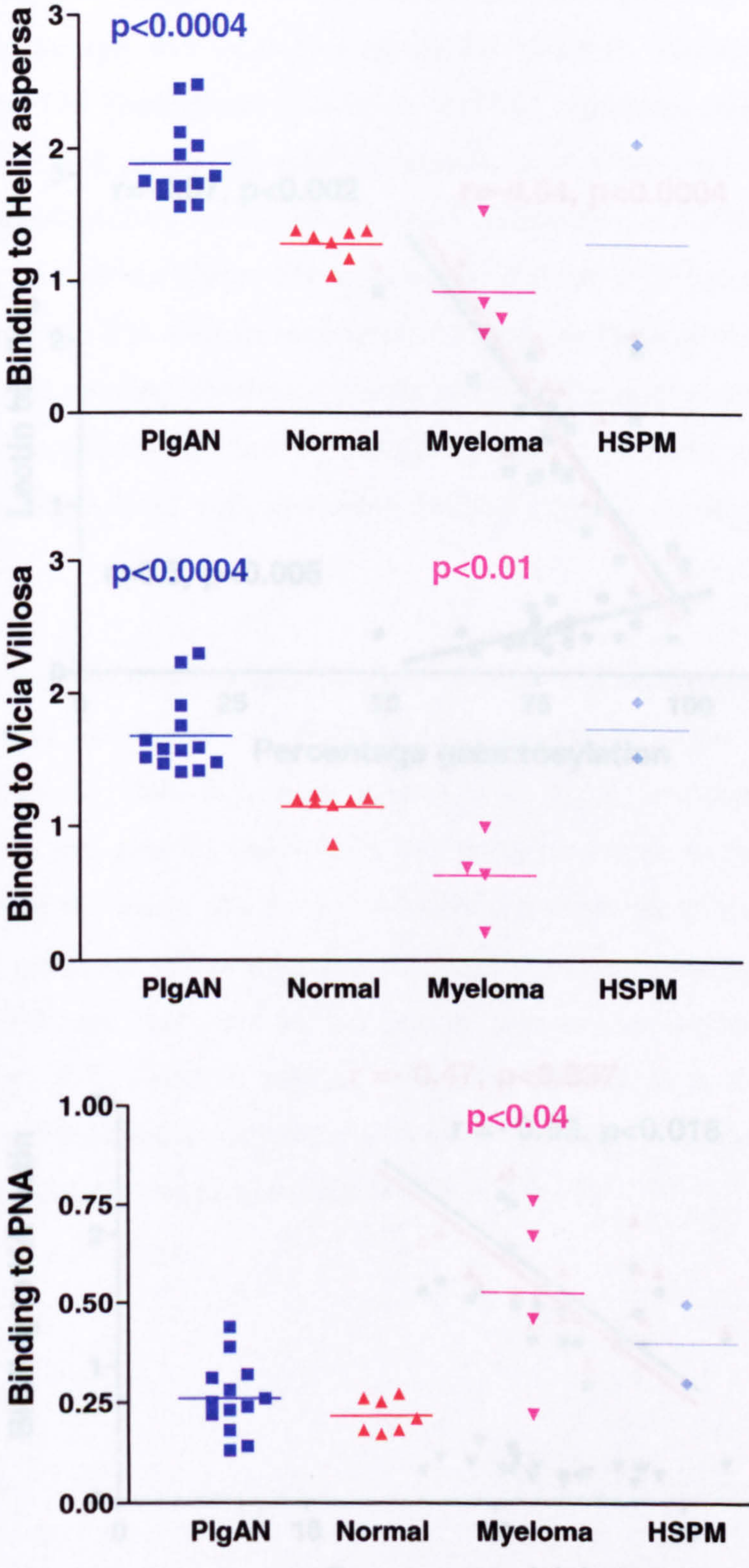
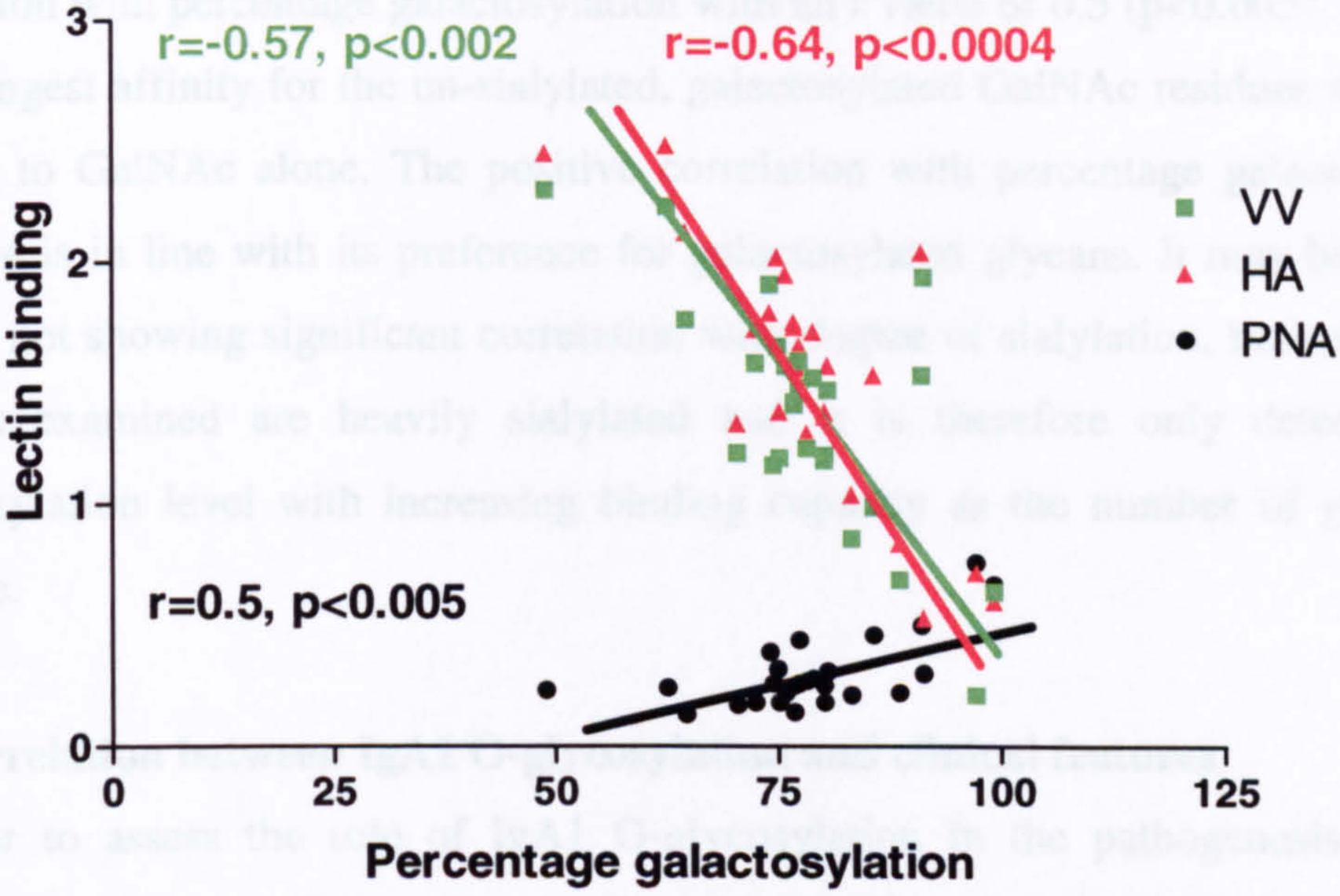



**Figure 5.17** Figures showing the differences in lectin binding between normal controls and patients with PIgAN, and myeloma. When analysed by the Mann Whitney U test, IgA from PIgAN shows significantly increased binding to HAA and VV and no difference in binding to PNA. Myeloma patients have increased binding to PNA and reduced binding to VV with no significant change in HAA binding.



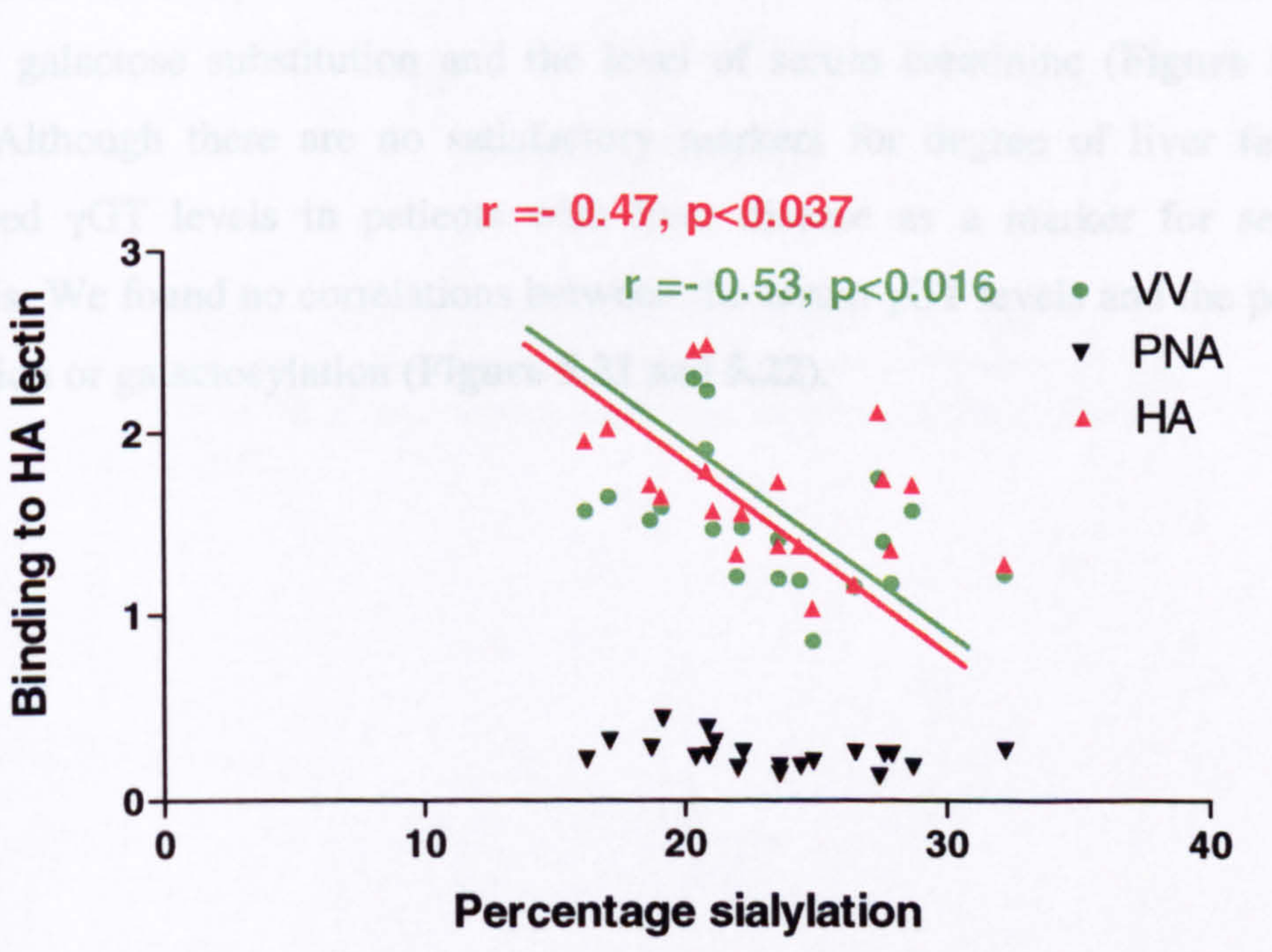




**Figure 5.18** Two graphs demonstrating the correlation between percentage galactosylation and sialylation with lectin binding. The top graph shows the negative correlation between VV and HA and number of galactose residues and a positive correlation with PNA. The lower graph shows that only VV and HAA binding correlate with the percentage sialylation and that there is no correlation with PNA.

strong negative charge that it carries. Therefore the more sialylated the hinge region, the more strongly will lectin binding will be inhibited. The results of the correlation between PNA binding and sialylation was not significant but there was a positive correlation with percentage galactosylation with an r value of 0.5 ($p<0.005$). PNA has the strongest affinity for the un-sialylated, galactosylated GalNAc residues with little binding to GalNAc alone. The positive correlation with percentage galactosylation therefore is in line with its preference for galactosylated glycans. It may be that the PNA is not showing significant correlation with degree of sialylation, because all the samples examined are heavily sialylated and it is therefore only detecting the galactosylation level with increasing binding capacity as the number of galactoses increase.

### 5.5 Correlation between IgA1 O-glycosylation and clinical features

In order to assess the role of IgA1 O-glycosylation in the pathogenesis and the progression of renal failure in patients with IgAN, we compared the degree of sialylation and galactosylation with the serum creatinine in the patient and controls enrolled in the study. We found no correlation between either the number of sialic acid or galactose substitution and the level of serum creatinine (**Figure 5.19 and 5.20**). Although there are no satisfactory markers for degree of liver failure, we employed γGT levels in patients with liver disease as a marker for severity of cirrhosis. We found no correlations between the serum γGT levels and the percentage sialylation or galactosylation (**Figure 5.21** and **5.22**).

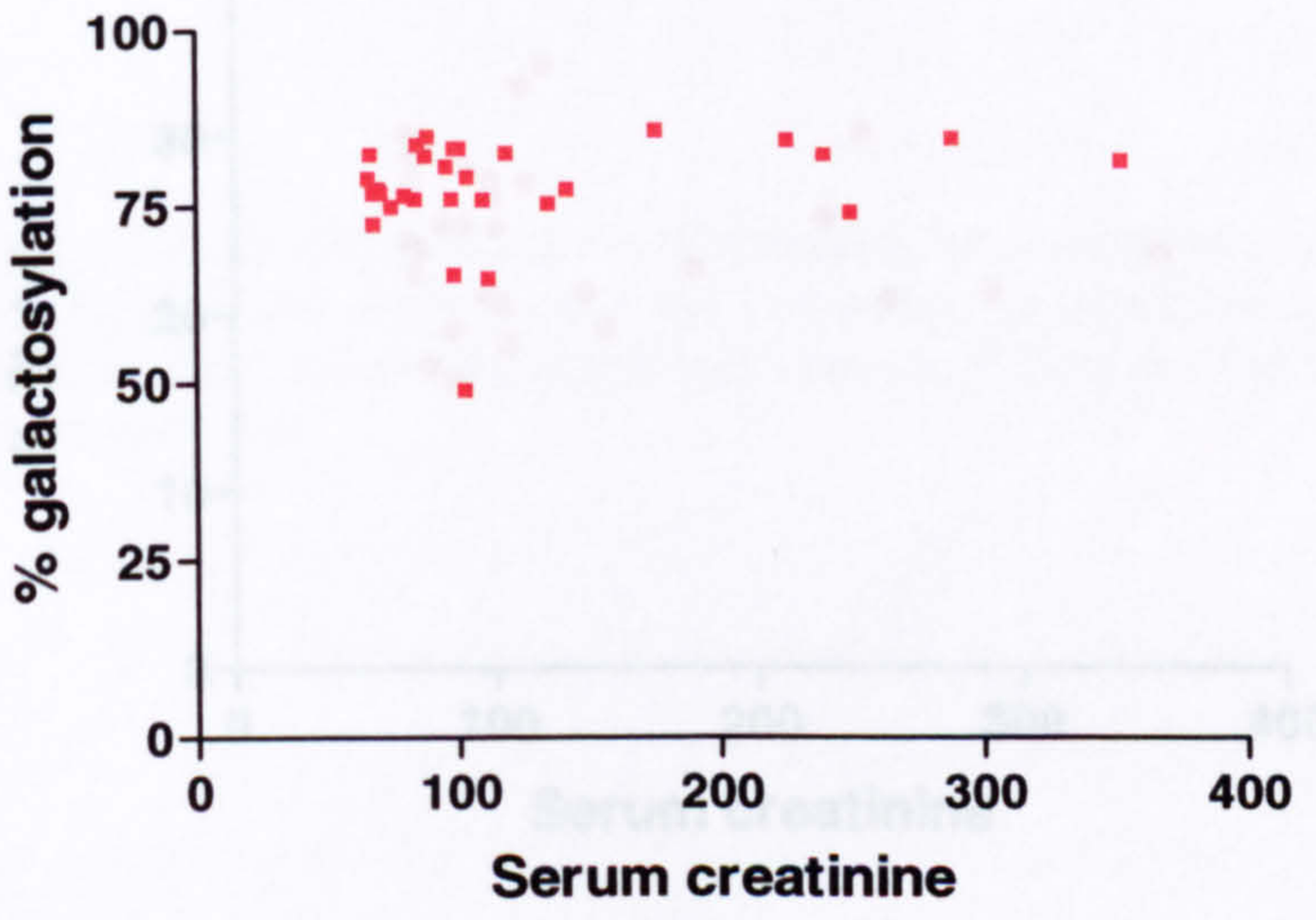



**Figure 5.19** This figure demonstrates that there is no correlation between the percentage galactosylation of the IgA1 HGP and serum creatinine in patients with primary IgAN.

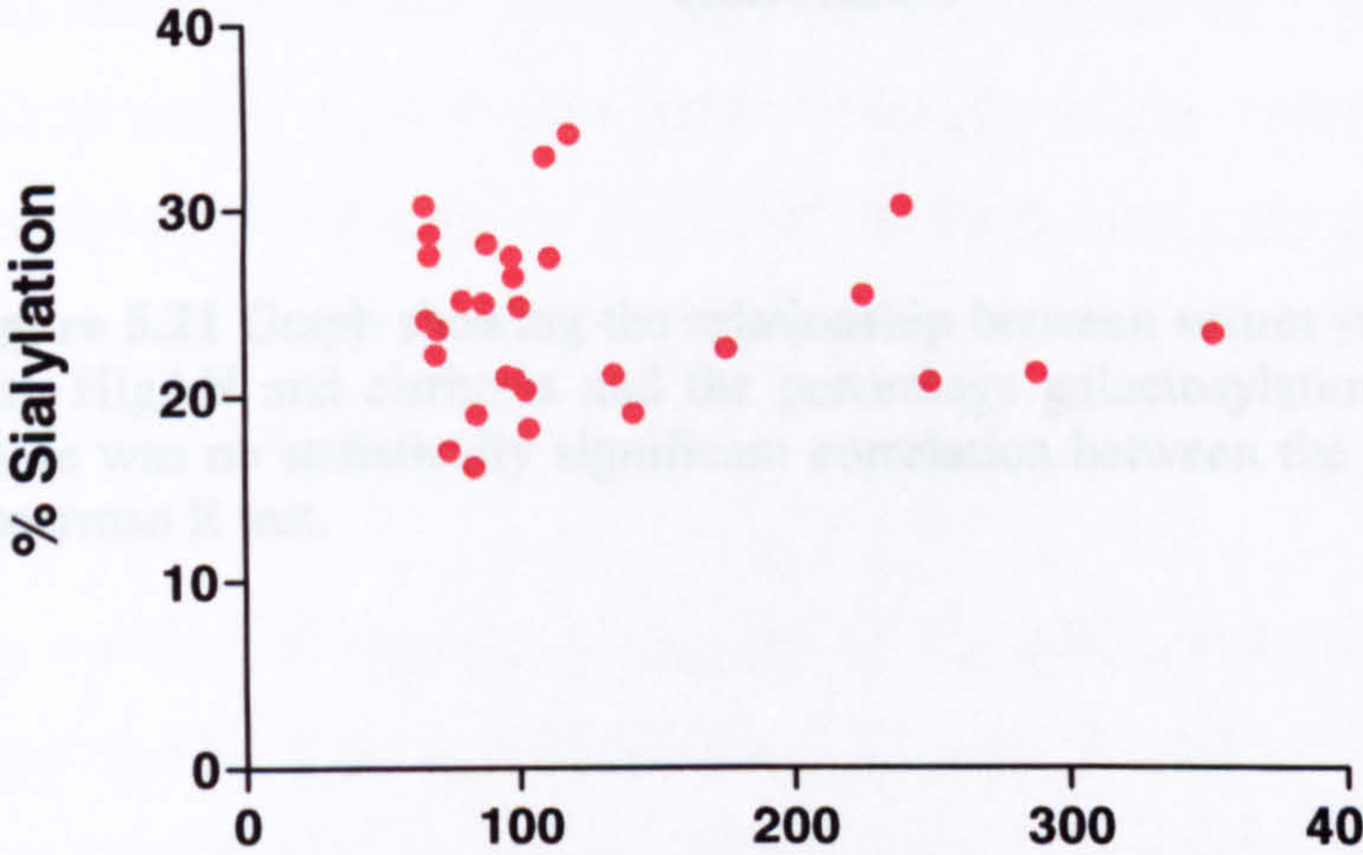



**Figure 5.20** This figure shows the absence of correlation between degree of sialylation and the serum creatinine in patients with primary IgAN.

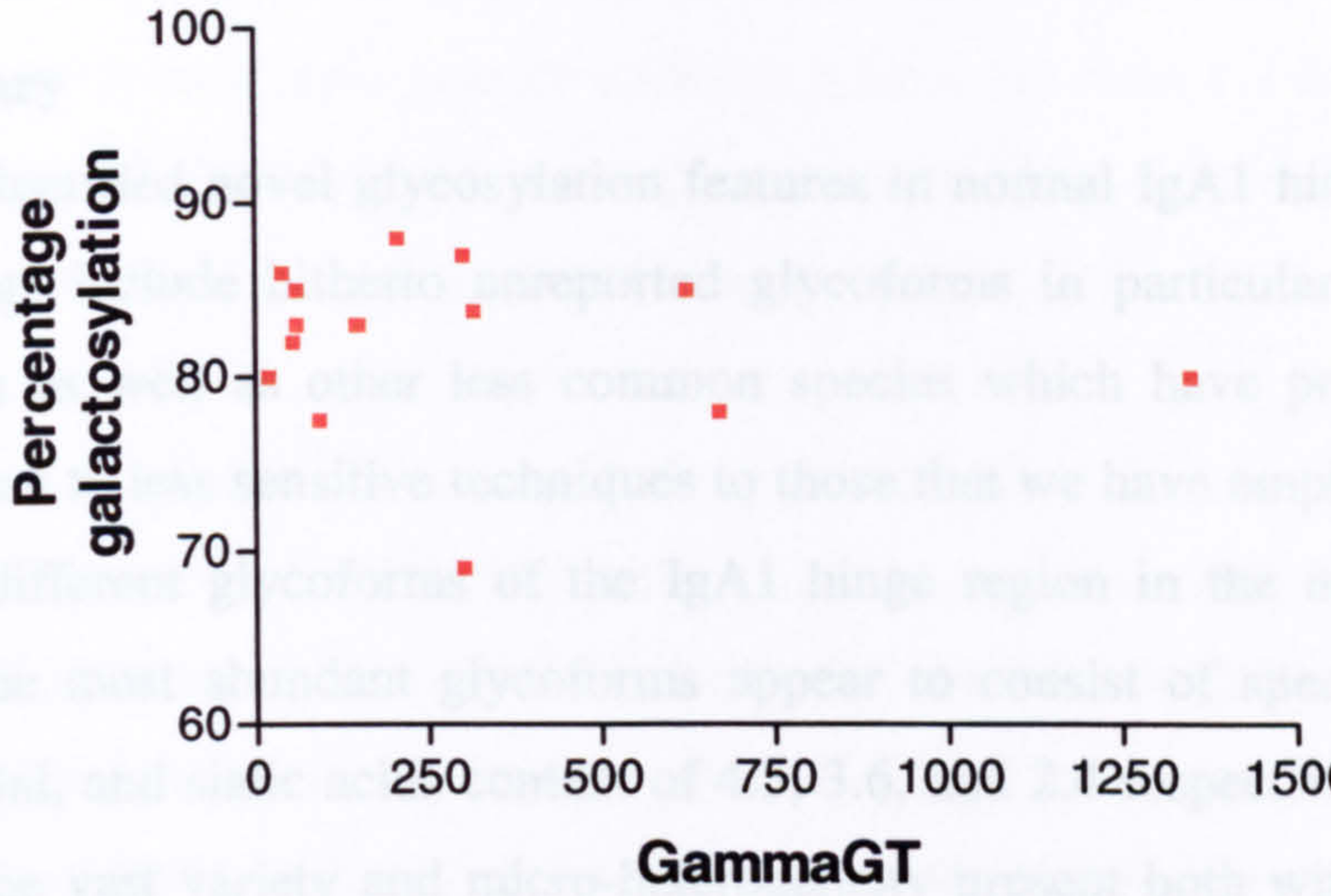



**Figure 5.21** Graph showing the relationship between serum γGT levels of patients with HIgAN and cirrhosis and the percentage galactosylation of the IgA1 HGP. There was no statistically significant correlation between the two parameters on a Spearman R test.

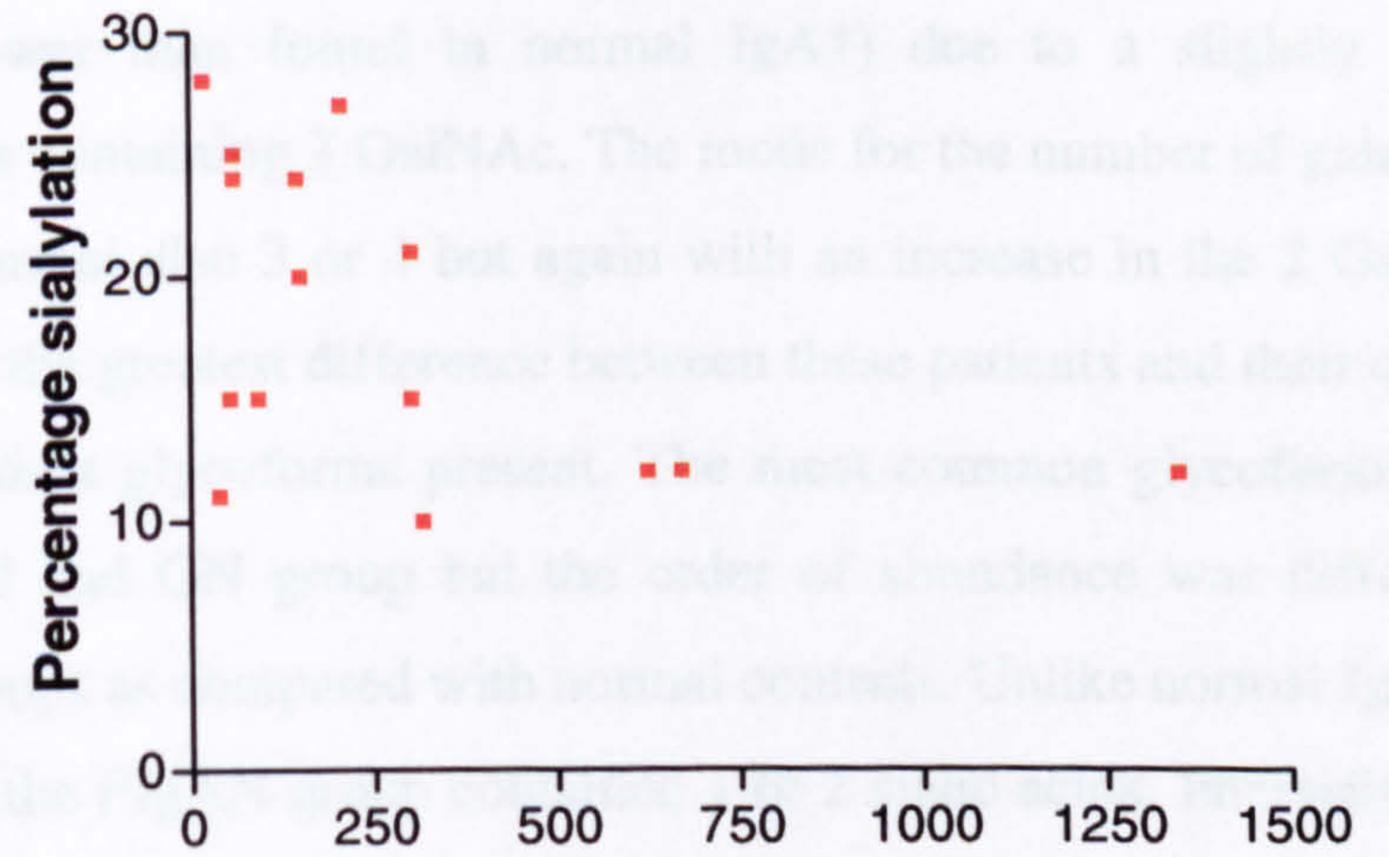



**Figure 5.22** Graph showing the relationship between serum γGT levels of patients with HIgAN and cirrhosis and the percentage sialylation of the IgA1 HGP. There was no statistically significant correlation between the two parameters on a Spearman R test.

## 5.6 Summary

We have identified novel glycosylation features in normal IgA1 hinge glycopeptide. Our findings include hitherto unreported glycoforms in particular the 6$^{th}$ GalNAc substitution as well as other less common species which have previously escaped detection due to less sensitive techniques to those that we have employed. In total we found 41 different glycoforms of the IgA1 hinge region in the normal population studied. The most abundant glycoforms appear to consist of species with a mean GalNAc, Gal, and sialic acids content of 4.5, 3.6, and 2.4 respectively. These results highlight the vast variety and micro-heterogeneity present both within the serum of each individual and in the collective sera of the group as a whole. When comparing the overall sialylation and galactosylation of the IgA1 hinge peptide in normal serum with that extracted from breast milk (mucosal IgA1), we found no significant differences between the two.

We found a number of significant changes in the glycosylation pattern of patients with primary IgAN. The spectra pointed towards global under-glycosylation in patients with PIgAN with a greater preponderance of species with lower GalNAc substitutions, minor reduction in galactosylation and significantly lower degrees of sialylation. These patients had a mean GalNAc number of 4.4 as their norm (which is slightly lower than found in normal IgA1) due to a slightly higher number of glycoforms containing 3 GalNAc. The mode for the number of galactose residues was like the normal also 3 or 4 but again with an increase in the 2 Gal species. We also found that the greatest difference between these patients and their controls were in the least abundant glycoforms present. The most common glycoforms were common to the normal and GN group but the order of abundance was different in both these patient groups as compared with normal controls. Unlike normal IgA1, the majority of species in the PIgAN group contained 1 or 2 sialic acids. Interestingly, these changes were not found to be exclusive to IgA nephropathy and patients with other types of GN were found to a lesser degree to be under-sialylated, but with no change in the pattern of galactosylation or number of GalNAc residues.

Patients with hepatic IgAN were found to be deficient in GalNAc residues, greatly under-sialylated and have slightly increased galactosylation which was statistically

significant depending on the statistical test used. Likewise patients with cirrhosis and no glomerular disease were grossly under-sialylated but were no different to normal where number of GalNAc residues was concerned. They appeared to have slightly increased galactose levels compared with normals.

We found no significant differences in the glycosylation of IgA1 in patients with HSP secondary to myeloma but this was perhaps due to the very small number of samples available for analysis. The myeloma IgA1 O-glycosylation demonstrated a number of features which distinguished it from the normal. Firstly, there was a tendency towards increased 3 GalNAc residues and an absence of 6 GalNAc found. Secondly more species with high galactose content (4 and 5) were observed and in the calculation of the overall percentage galactosylation, this was found to be significantly increased. Finally we saw no consistent changes in sialylation in this group of patients. There was a wide scatter around the mean and the small numbers made any meaningful comparison with the other groups invalid.

An important technical point reflected in our data is that our analyses using mass spectrometry on purified IgA1 correlate well with results of lectin binding studies performed on serum. This suggests that our MS findings on purified IgA1 are representative of the whole array of glycoforms present in the serum and that no major glycoforms have been lost in the processing of the samples using jacalin affinity chromatography.

## 5.7 Conclusions

We have found great variety in the normal glycoforms of the IgA1 hinge glycopeptide using MALDI mass spectrometry. Our results confirm the previous findings of other groups that the O-glycosylation changes in PIgAN are real. We have not only found evidence of under-sialylation, but also slight reduction in galactosylation and changes in the GalNAc content of the HGP in primary IgAN. However, the finding of similar changes in patients with other GNs suggests that under-glycosylation is not specific to PIgAN and therefore probably not necessarily a sole factor in its pathogenesis. We also found the main differences between the patient and control groups in the least abundant glycoforms. This may mean that such unusual glycoforms are the pathogenic species involved in the deposition of IgA1 and triggering of an

inflammatory process. Further studies of these 'micro-glycoforms' are required to assess their pathogenicity.

IgA1 O-glycosylation of patients with HIgAN and cirrhosis had not previously been studied by mass spectrometry. We found that these patients had a marked degree of under-sialylation. This could be explained by the poor hepatocyte function in this patient group. Failure of the ASGPR to clear serum IgA can result in high levels of circulating de-sialylated IgA1 in these patients. Given that this finding was common to both patients with and without nephropathy, it is unlikely that under-sialylation of IgA1 per se leads to the development of renal disease. Also the mechanism of decreased GalNAc substitutions and increased overall galactosylation in HIgAN cannot be explained and contradicts the finding in the PIgAN literature where under-galactosylation has been proposed as the key pathogenic factor in IgA1 deposition. The absence of these changes in patients with cirrhosis without renal disease suggests they are not a simple consequence of the 'cirrhotic milieu'.

Finally, the changes found in patients with myeloma are difficult to explain but are probably a reflection of the properties of the specific clonal expansion for each individual. None the less, the increased galactosylation appears to be a real phenomenon and further studies in this area may shed light on the post-translational modification mechanisms controlling IgA1 HGP glycosylation.

In conclusion, our study has clearly demonstrated the presence of a variety of glycosylation changes in the different patient and control groups. No obvious pattern or candidate glycoform has been identified as one that leads to the development of IgAN. The presence of a vast variety of glycoforms in different conditions poses more questions about the significance of glycosylation patterns in different disease processes rather than providing a key to the aetiology of IgAN.

# Chapter 6: Fcα-Receptor I (CD89) mediated IgA clearance

## 6.1 Introduction

Monocyte and neutrophil FcαR1 (CD89) expression was measured in these studies as per protocols discussed in the methodology chapter (3). The aim of these studies was to observe any differences in the expression of the CD89 receptor on circulating monocytes and neutrophils in our patient and control groups. Previous reports in this area have been contradictory some showing increased (Kashem et al 1994; Kashem et al 1996) and others showing decreased receptor expression in IgA nephropathy (Grossetette et al 1998). These findings have been used by some to explain the elevated IgA levels in patients with IgAN and liver disease (Silvain et al 1995). Using cells obtained from the patients and controls described in chapter one, we examined the expression of this receptor on circulating white blood cells.

## 6.2 Flow cytometry

Polymorph mononuclear cells were obtained as described in chapter 3 and stained with the relevant positive and negative control antibodies at 4°C to avoid modulation in the receptor expression. FACS analysis results were obtained as forward and side scattered profiles. The median channel fluorescence (MCF) was used as the measure of cell fluorescence intensity because this parameter has been shown to most accurately reflect the average fluorescence of cells in a cytometric sample. In all cases the data presented have been corrected for non-specific binding by subtraction of auto-fluorescence and background staining. Fluorescence intensity was analysed with log amplifier output and light scatter data acquired in the linear mode. Forward scatter (FSc) and side scatter (SSc) were measured and data stored in list mode. **Figure 6.1** shows the forward and side scatter characteristics of whole blood. U937 cells, which are an immortalised monocytic cell line were also used as a positive control for the presence of CD89 activity. Cells were taken from the 3$^{rd}$ passage onwards. The forward and side scatter profiles for these cells are demonstrated in **Figure 6.2.** Furthermore, the shift in the cells is demonstrated in **Figure 6.3** where the U937 cells are counterstained with anti-CD89 antibodies. The cells were all stained with antibodies to confirm the characteristics of the populations being examined. **Figure**

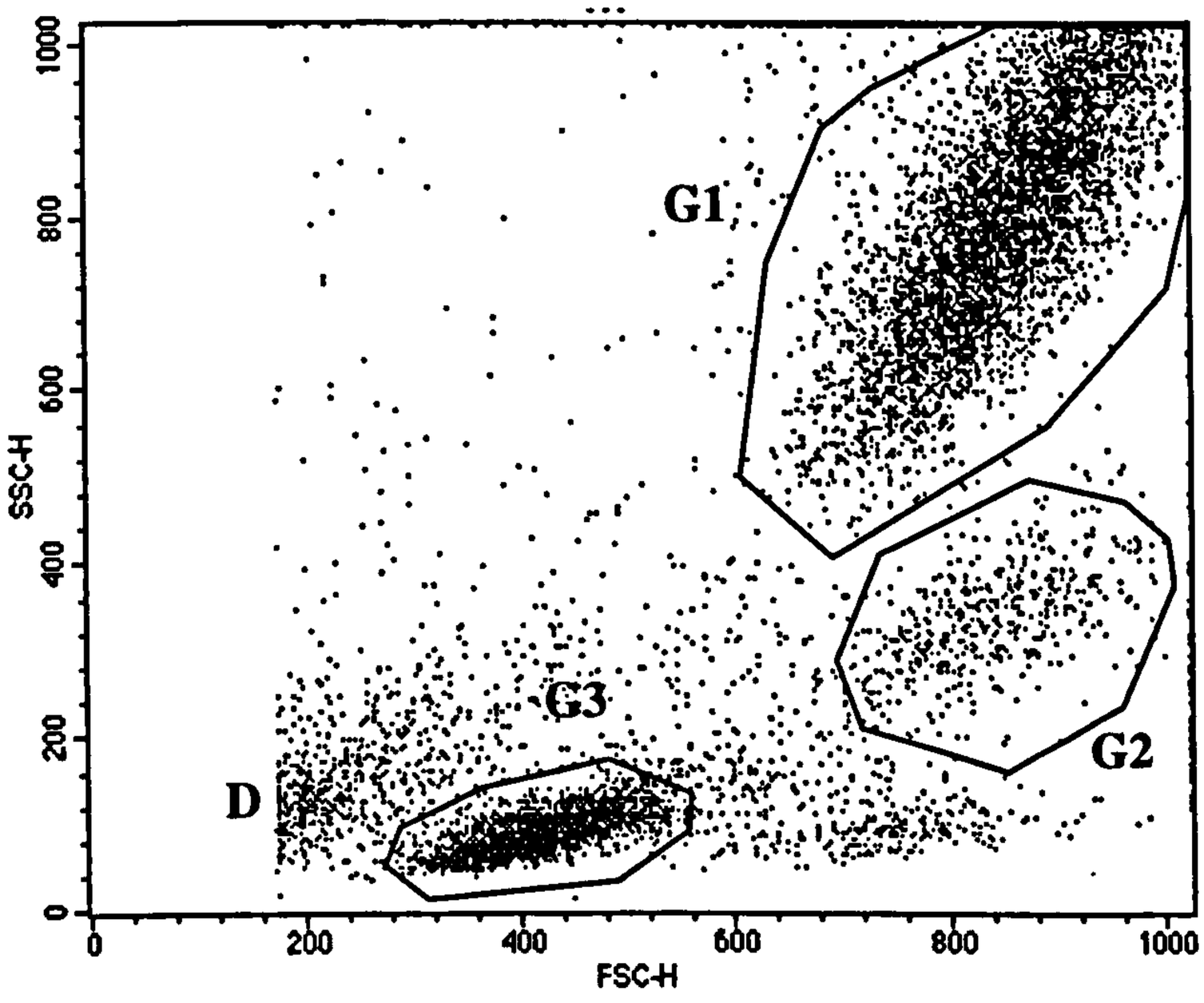



**Fig 6.1 Forward scatter and side scatter characteristics of white blood cells after separation from whole blood.** Gates were established around the separate populations of neutrophils (G1), monocytes (G2) and lymphocytes (G3). These gates were used for live gating during data acquisition. Dead cells and cell debris can be seen occupying the lower left hand corner of the plot (D).

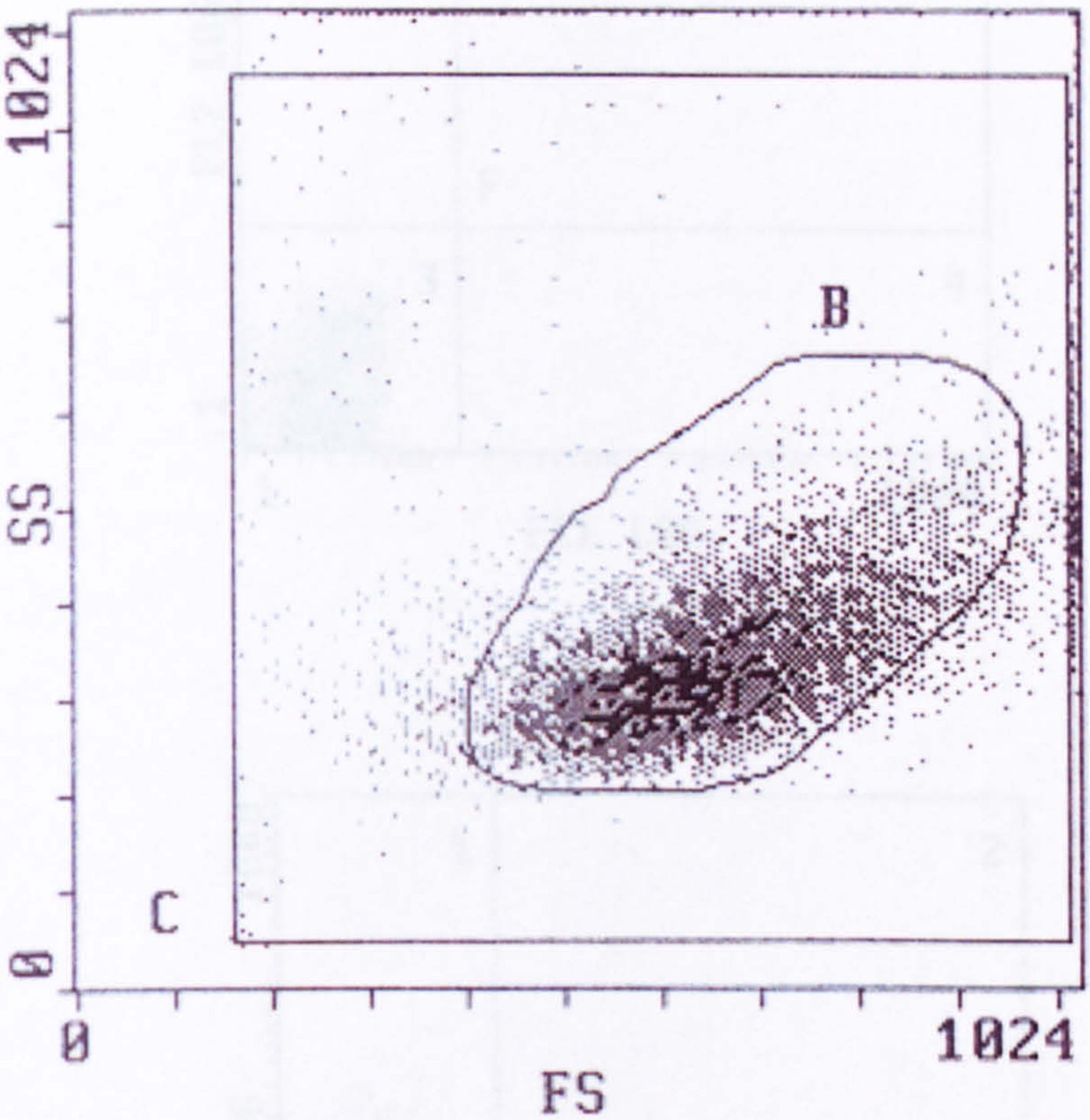
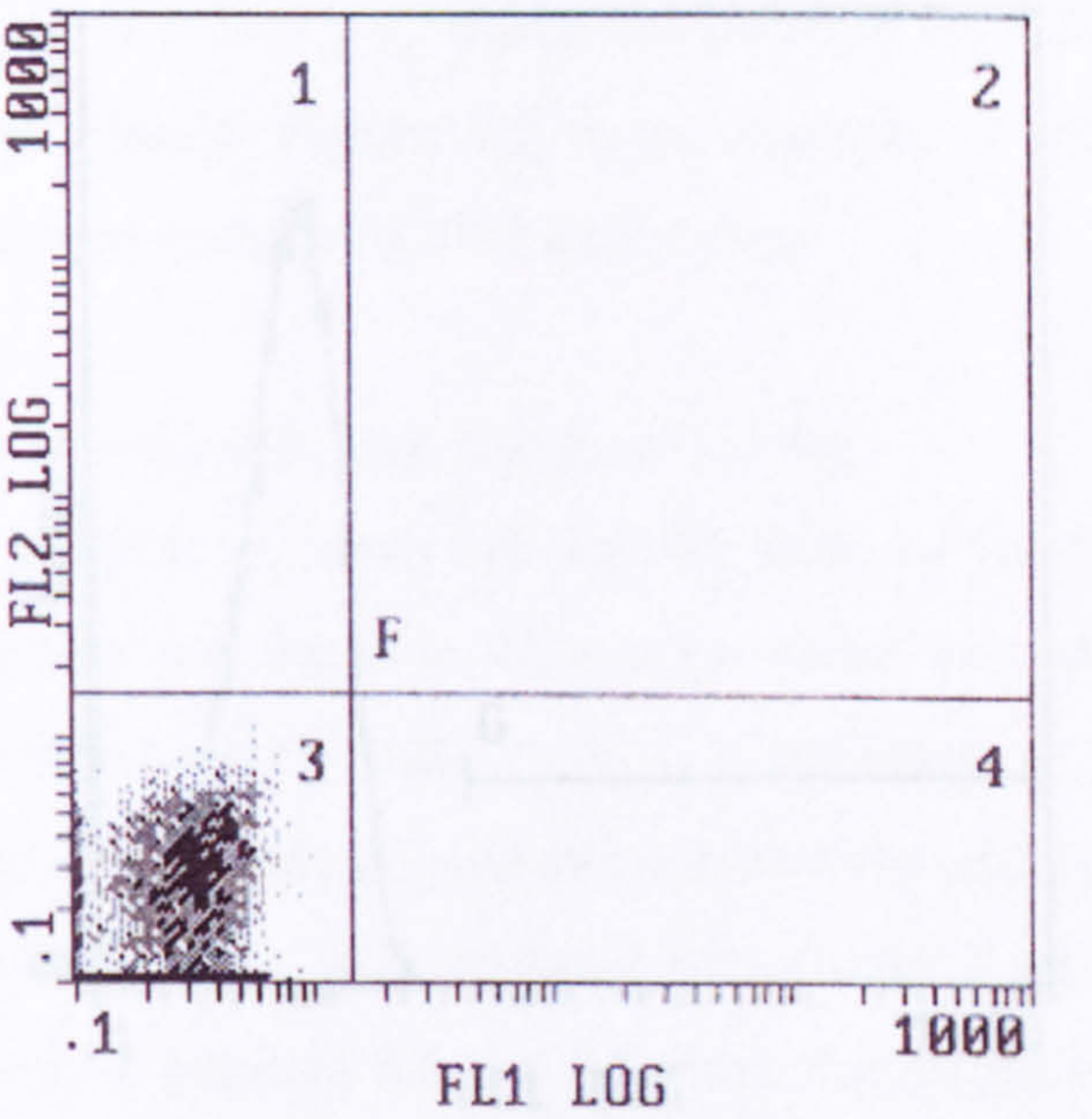



**Figure 6.2 U937 cells scatter characteristics.** Acquisition gates were drawn around homogeneous cell populations and data live-gated for all experiments. There was no appreciable change in the gating during the course of the studies.



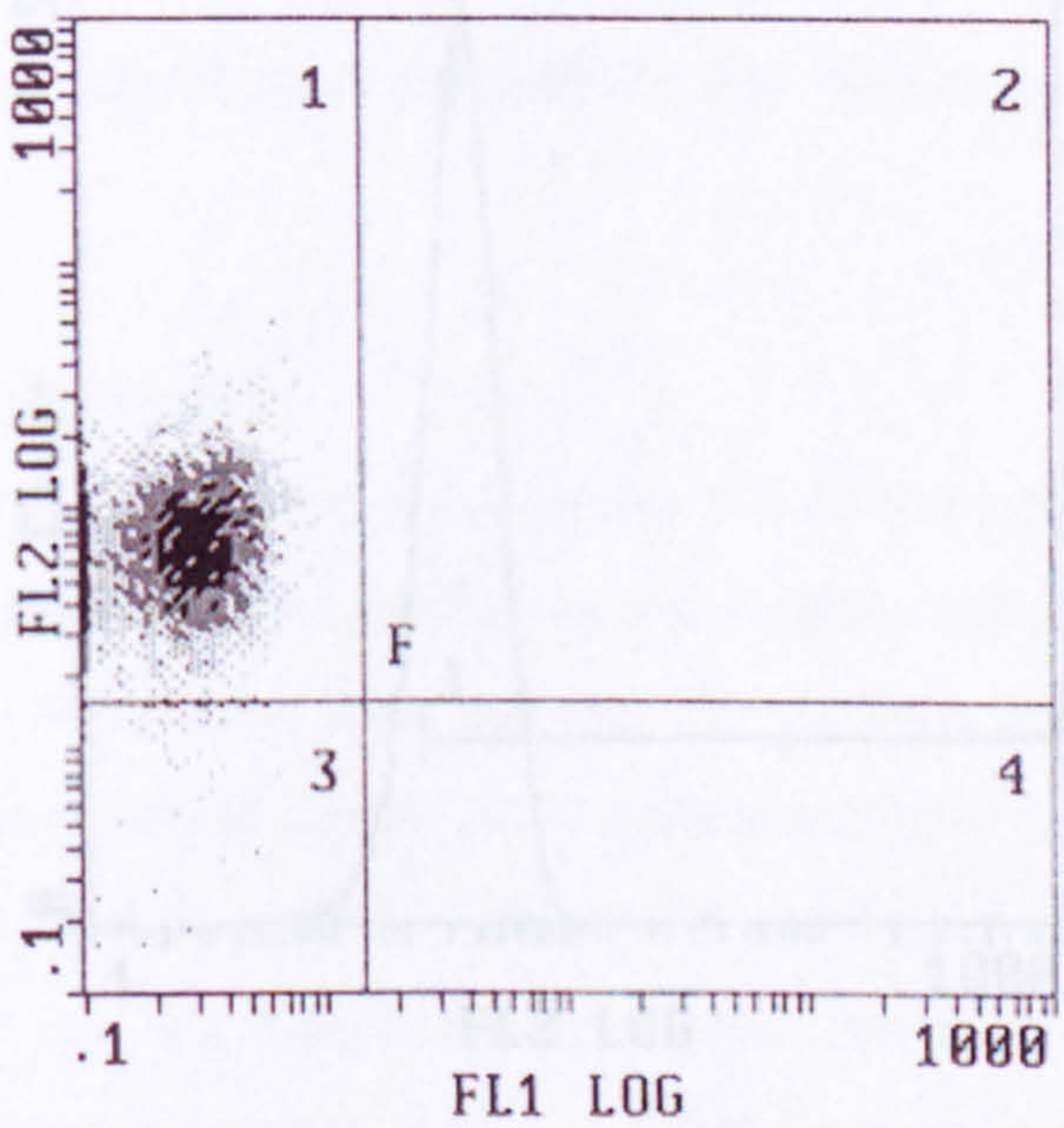




**Figure 6.3** Shows the shift in the Fc-block stained U937 cells before (top figure) and after (bottom figure) staining with anti-CD89 antibody. This demonstrates that all the cells express CD89 on their surface and may be used as a positive control for the peripheral blood mononuclear cells under study.

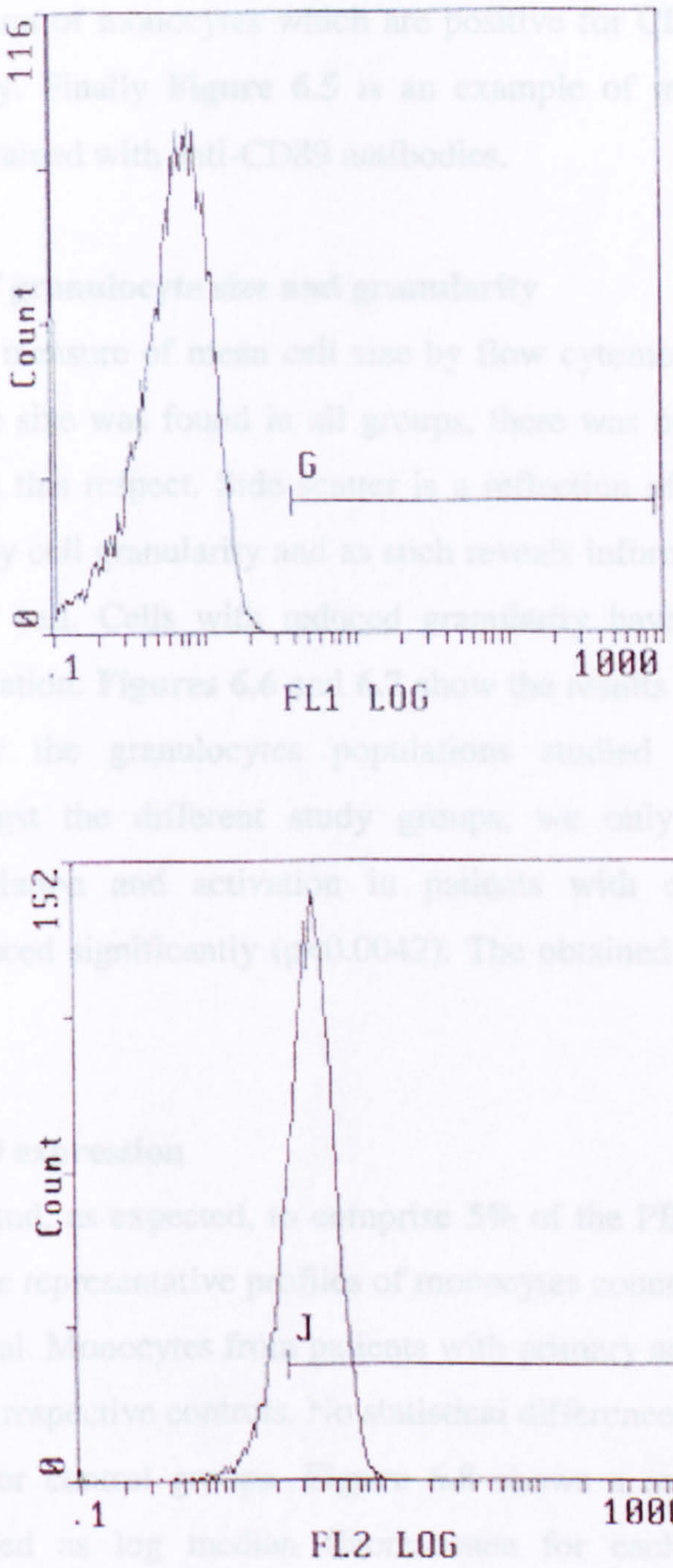



**Figure 6.4** Shows the shift in the population of monocytes stained with Fc block only in the top figure. The shift shown in the cells in the lower trace is after double staining with anti-CD14 antibody, which is specific to monocytes, confirming the presence of monocytes in the sample under study.

**6.4** shows a population of monocytes which are positive for CD14 when stained by the relevant antibody. Finally **Figure 6.5** is an example of monocytes showing a positive shift when stained with anti-CD89 antibodies.

### 6.3 Measurement of granulocyte size and granularity

Forward scatter is a measure of mean cell size by flow cytometry. Although a wide range of granulocyte size was found in all groups, there was no difference between their mean values in this respect. Side scatter is a reflection of cellular complexity. This is determined by cell granularity and as such reveals information about the state of activation of the cell. Cells with reduced granularity have been activated and undergone de-granulation. **Figures 6.6** and **6.7** show the results of the mean side and forward scatter for the granulocytes populations studied by flow cytometry respectively. Amongst the different study groups, we only found evidence of neutrophil de-granulation and activation in patients with cirrhosis, where cell granularity was reduced significantly ($p<0.0042$). The obtained data are summarised in **Table 6.1.**

### 6.4 Monocyte CD89 expression

Monocytes were found, as expected, to comprise 5% of the PBMCs in the samples. **Figure 6.4** shows the representative profiles of monocytes counter-stained with CD14 in a normal individual. Monocytes from patients with primary and hepatic IgAN were compared with their respective controls. No statistical differences were found between any of the patient or control groups. **Figure 6.8** shows a scatter graph of CD89 expression, expressed as log median fluorescence for each subject group. No significant difference was apparent in the mean value for each group.

### 6.5 Neutrophil CD89 expression

The same analyses were performed to assess the CD89 expression on neutrophils. Again we did not find any significant differences between normal controls and the patient groups and their respective controls. **Figure 6.9** illustrates these results.

### 6.6 Percentage CD89 positive monocytes

In order to assess the contribution of monocyte numbers to the above results, we assessed the percentage of monocytes, which were CD89 positive by gating on the

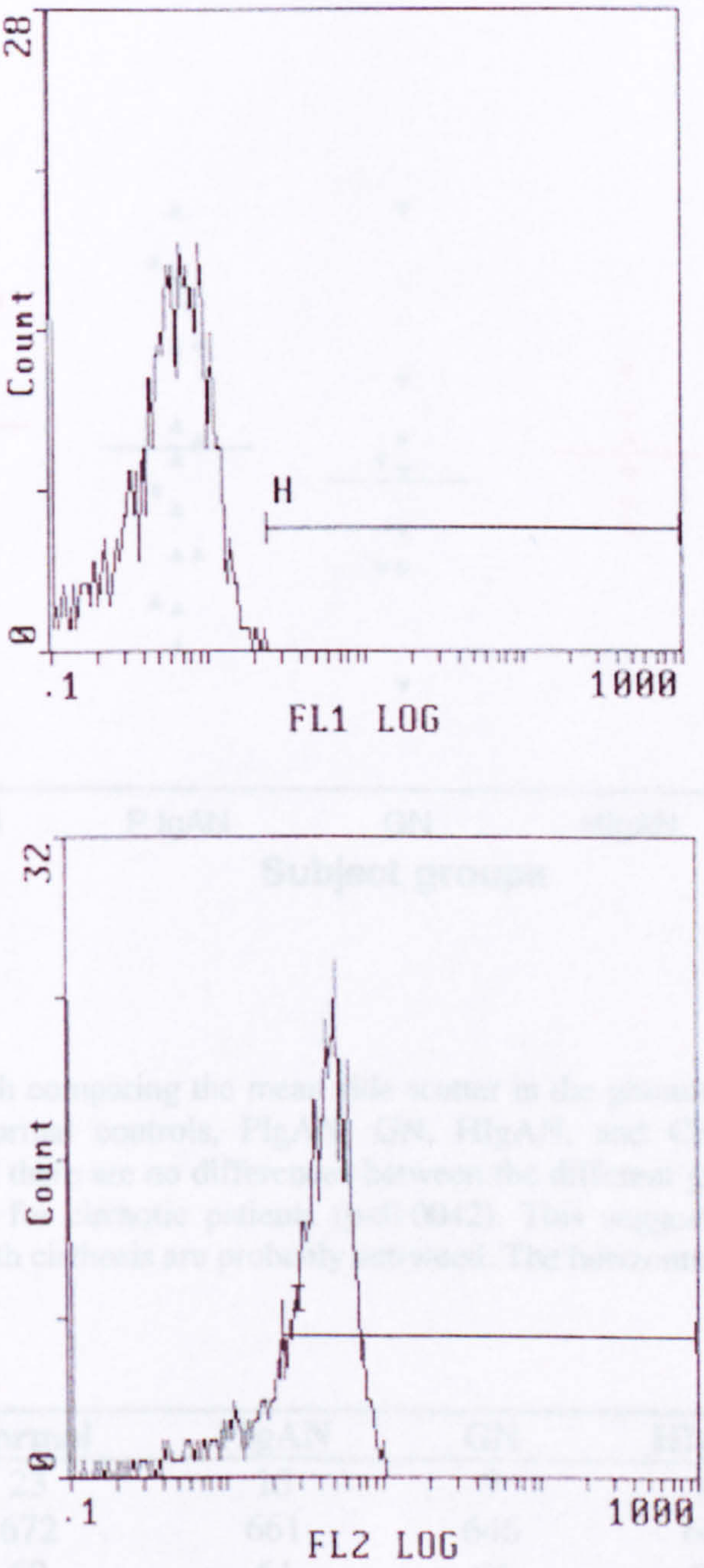



**Figure 6.5** Shows an example of the shift in the population of monocytes stained with Fc block alone in the top figure. The shift shown in the cells in the lower trace is after double staining with anti CD89 antibody.

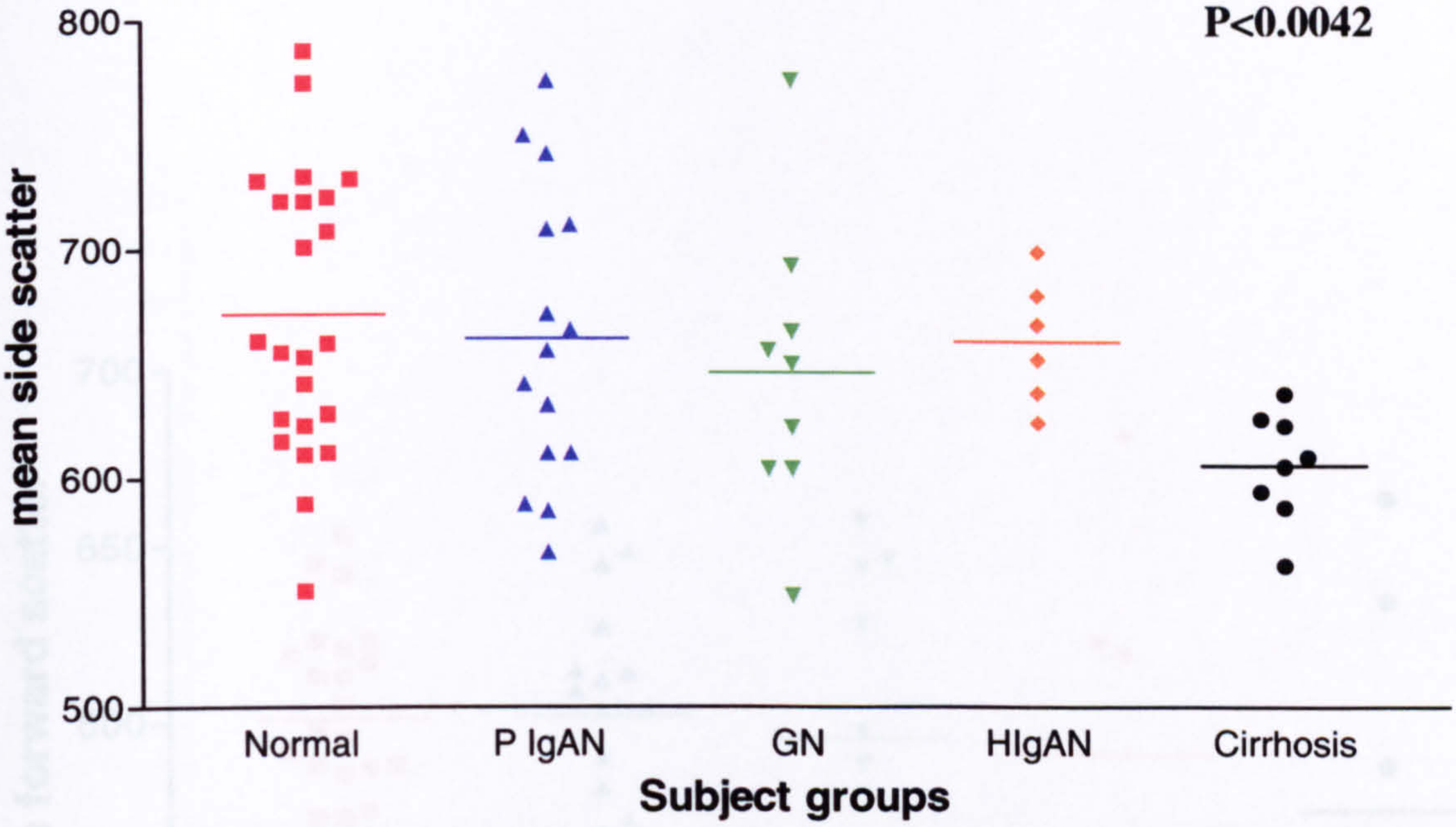



**Figure 6.6** Graph comparing the mean side scatter in the granulocytes studied by flow cytometry in normal controls, PIgAN, GN, HIgAN, and Cirrhosis. These results demonstrate that there are no differences between the different groups in granularity of the cells except for cirrhotic patients ($p<0.0042$). This suggests that the neutrophils from patients with cirrhosis are probably activated. The horizontal bar is the mean value for the group.

| | Normal | PIgAN | GN | HIgAN | Cirrhosis |
|---|---|---|---|---|---|
| **No.** | 23 | 15 | 9 | 6 | 8 |
| **Mean** | 672 | 661 | 646 | 660 | 605 |
| **SD** | 62 | 64 | 64 | 28 | 24 |
| **SE** | 13 | 17 | 21 | 11 | 8.6 |
| **P value** | N/A | 0.68 | 0.28 | 0.73 | **0.0042*** |
| **MWU** | N/A | 158 | 77 | 62 | 28 |

**Table 6.1** Table summarising the statistical analyses of the side scatter data. The Mann-Whitney U Test (MWU) was used as a non-parametric statistical tool in these analyses.

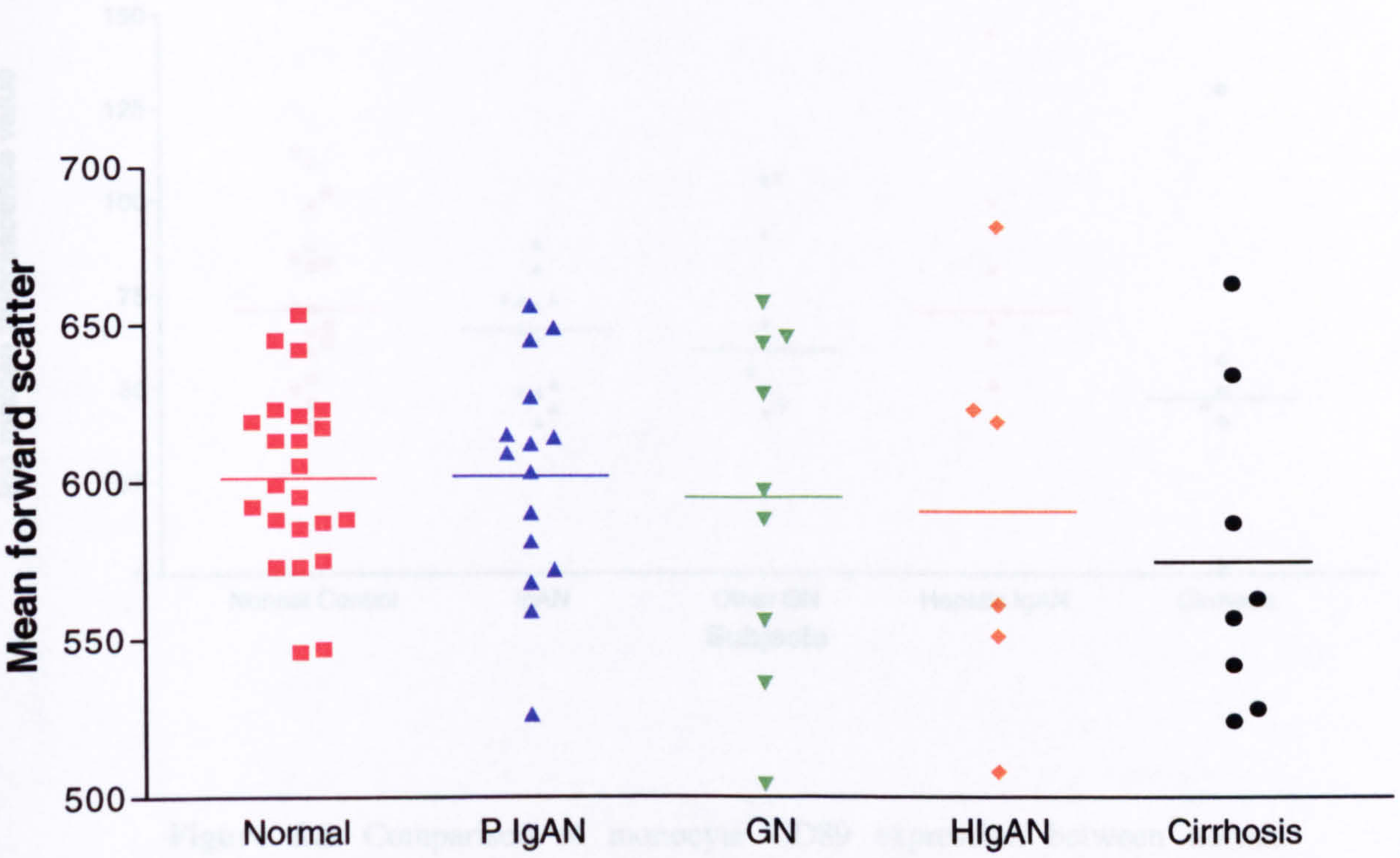



**Figure 6.7** Mean granulocytes forward scatter in the different patient groups are shown in this graph. There was no difference between the groups indicating the absence of any significant difference in size between granulocytes in the different patients and their controls.

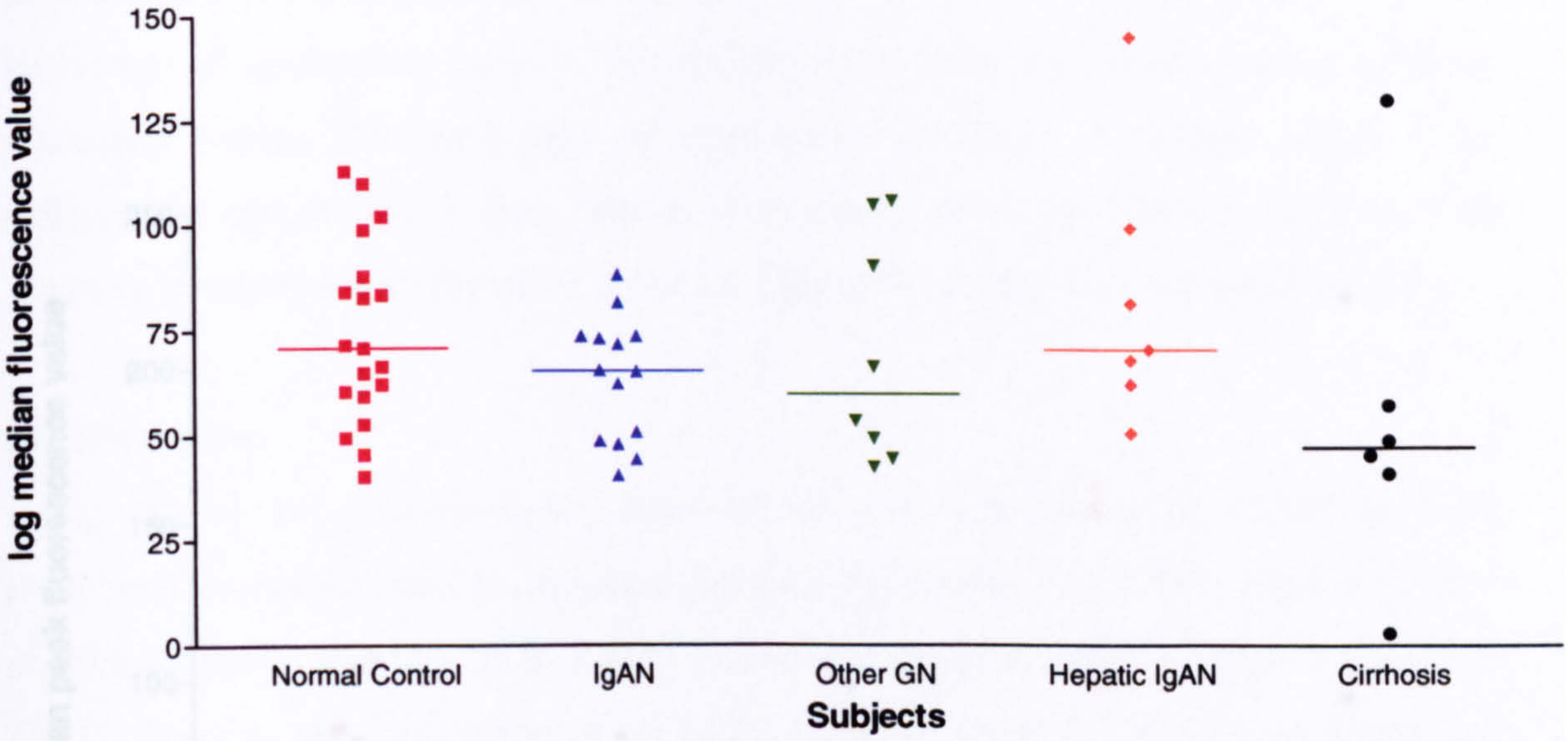



**Figure 6.8.** Comparison of monocyte CD89 expression between normal controls (n=19), patients with primary IgAN (n=14), other primary glomerulonephritides (other GN, n=8), hepatic IgAN (n=7), and cirrhosis (n=6) No significant difference was found in FcαR1 expression (ordinate) between the different subject groups. The horizontal bars indicate the mean for each group.

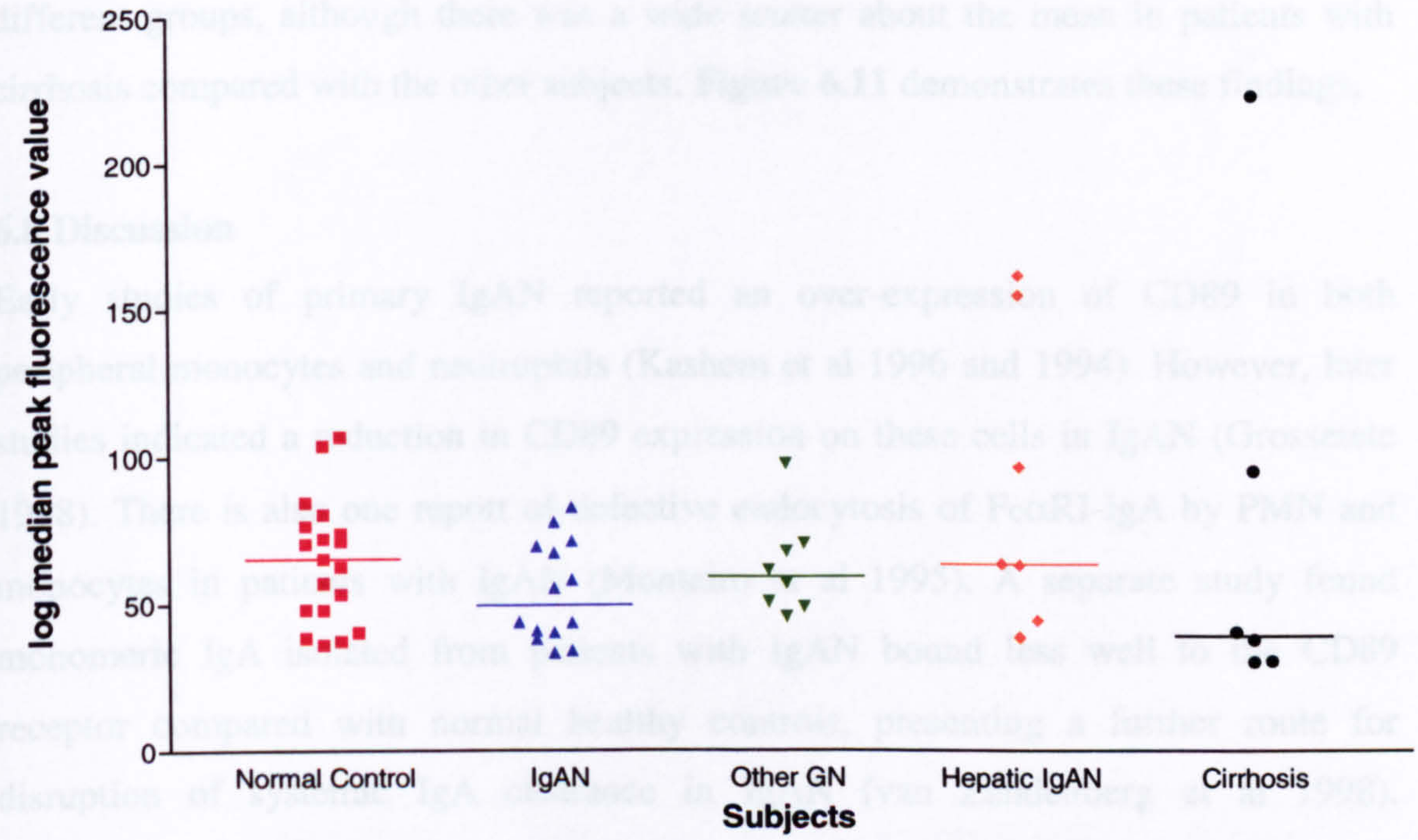



**Figure 6.9** CD89 surface expression on neutrophils compared in normal controls, patients with primary IgAN, patients with other GN, hepatic IgAN, and cirrhosis. As with monocyte CD89 expression, no difference was found to be statistically significant between the groups.

monocytes when stained with the CD89 antibody. We illustrated no differences between any of the groups as seen in **Figure 6.10**. This suggests that the CD89 expression is uniform within the monocyte population.

### 6.7 Percentage CD89 positive neutrophil

Numbers of neutrophil positive for CD89 were assessed by the same methods discussed above. The percentage of these cells was fairly consistent between the different groups, although there was a wide scatter about the mean in patients with cirrhosis compared with the other subjects. **Figure 6.11** demonstrates these findings.

### 6.8 Discussion

Early studies of primary IgAN reported an over-expression of CD89 in both peripheral monocytes and neutrophils (Kashem et al 1996 and 1994). However, later studies indicated a reduction in CD89 expression on these cells in IgAN (Grossetete 1998). There is also one report of defective endocytosis of FcαRI-IgA by PMN and monocytes in patients with IgAN (Monteiro et al 1995). A separate study found monomeric IgA isolated from patients with IgAN bound less well to the CD89 receptor compared with normal healthy controls, presenting a further route for disruption of systemic IgA clearance in IgAN (van Zandenberg et al 1998). Alterations in the post-translational modification of FcαRI (CD89) have also been reported in IgAN. Myeloid FcαRI from these patients were found not only to be consistently larger (60 to 85 kDa) than those of controls (55 to 75 kDa) but also to have decreased binding to a sialic acid-specific lectin indicating impaired sialylation of surface FcαRI molecules (Grossetete et al 1998). This could potentially influence the receptor-ligand interaction and may help explain the reduced binding of monomeric IgA seen in IgAN.

Silvain et al (1995) have found reduced CD89 levels on monocytes in patients with alcoholic cirrhosis with a significant correlation between serum IgA levels and monocyte CD89 expression. Whilst there has been some evidence for defective monocyte function in patients with hepatic IgAN in the literature (Roccatello 1985), there are no studies of Fc alpha expression in hepatic IgAN. In this study we have

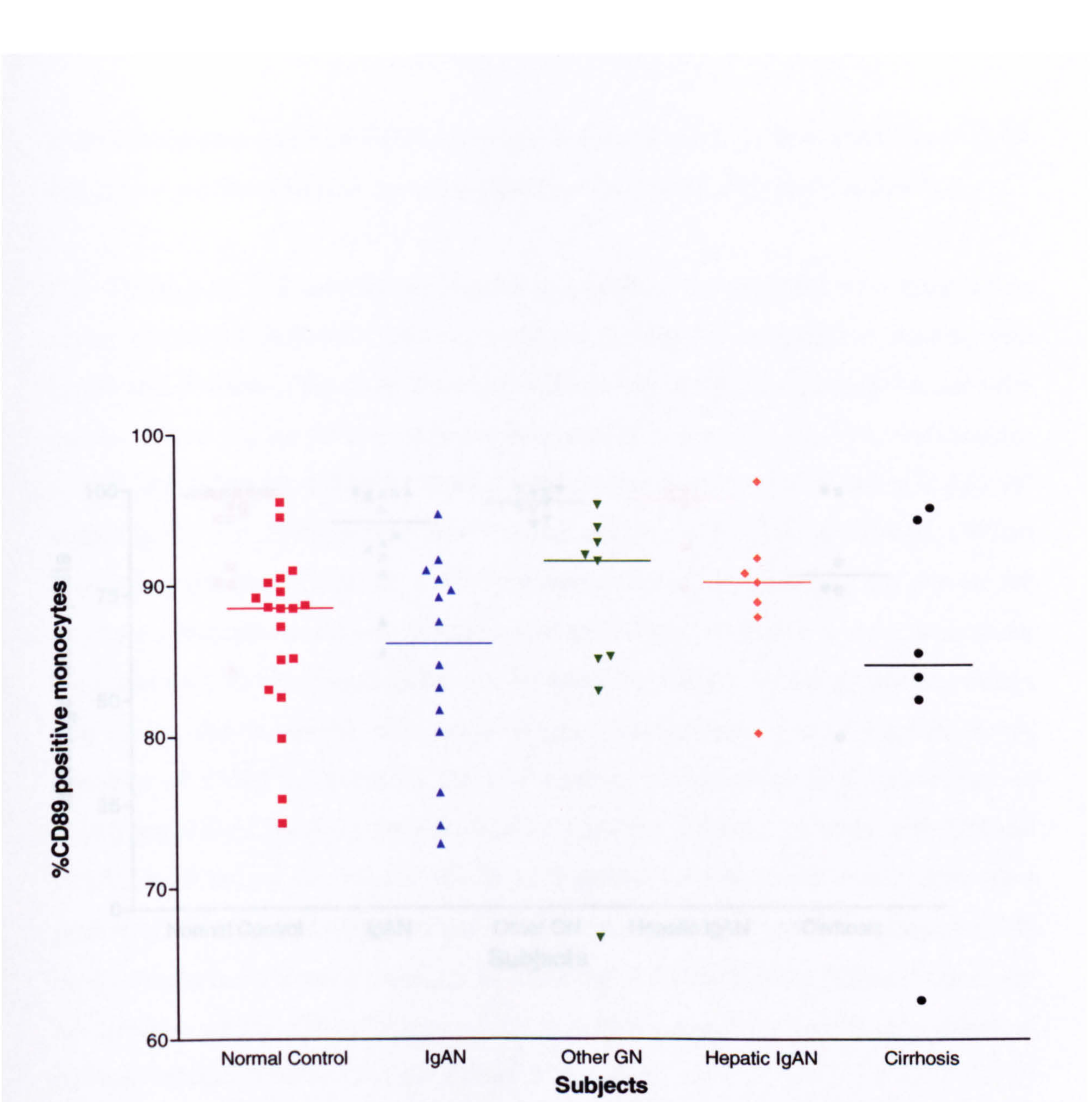



**Figure 6.10** Percentage of CD89 positive monocytes in samples taken from patients with primary IgAN, hepatic IgAN, other glomerulonephritides, cirrhosis, and normal controls; All different groups showed a wide spectrum of CD89 positivity about the mean. There was no significant difference found between the groups. The mean (horizontal bar) however does show a trend towards reduced numbers of cells with CD89 expression in both PIgAN and cirrhosis.

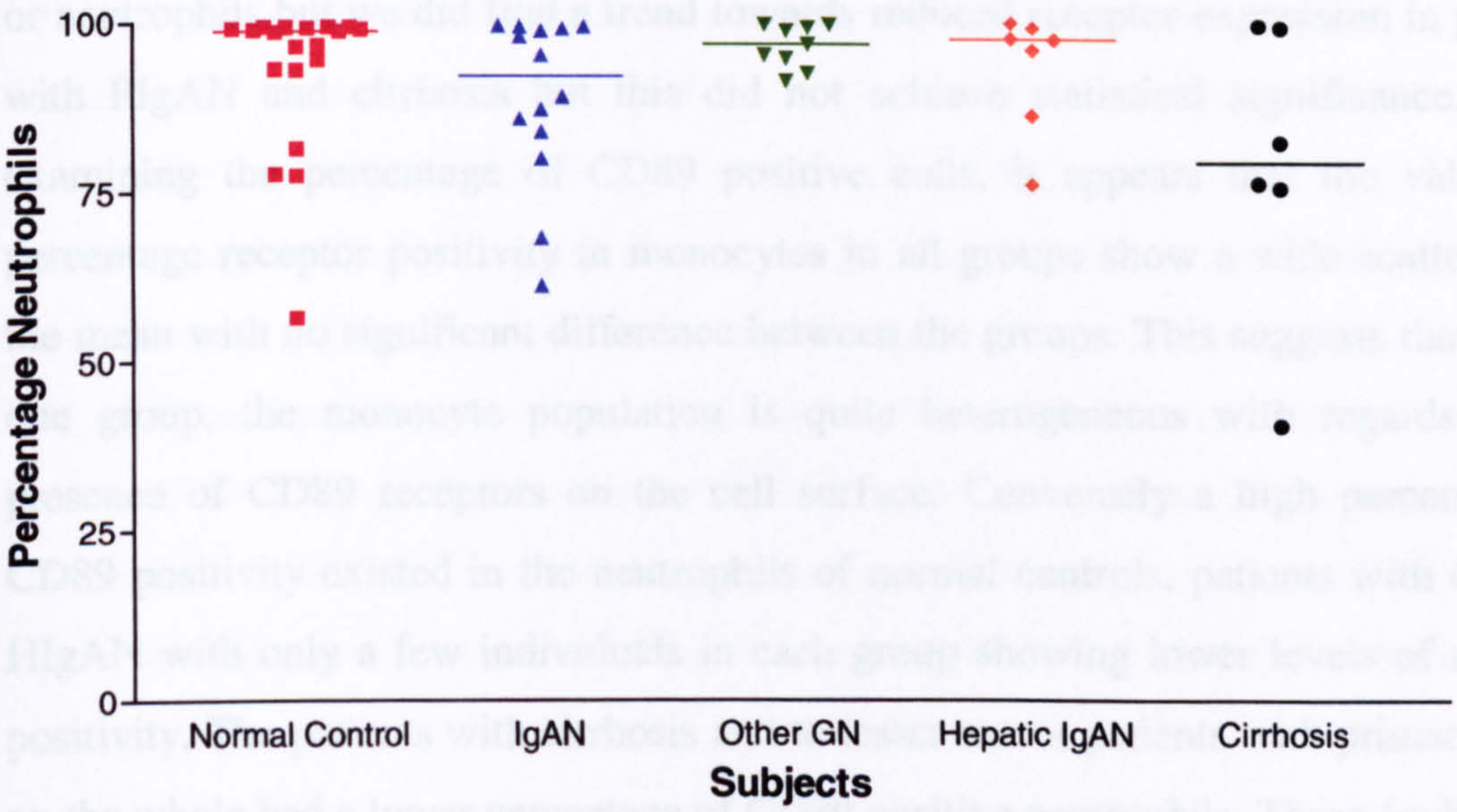



**Figure 6.11** Percentage of CD89 positive neutrophils compared in normal controls, patients with primary IgAN, patients with other GN, hepatic IgAN, and cirrhosis. As with % of CD89 positive monocytes no difference was found to be statistically significant between the mean values for the patient and control groups. The horizontal bar is the mean for the values obtained within each group and this shows that the percentage of CD89 positive neutrophils in patients with cirrhosis is reduced when compared with the median value for the other groups. This may be due to the activated state found in this group's granulocytes.

looked at the expression of CD89 receptors in patients with primary and hepatic IgAN and their controls as well as measuring the percentage of CD89 positive PBMCs.

Our findings do not support the results of previous investigators who have either shown elevated or reduced CD89 expression in peripheral blood cells of patients with IgAN and cirrhosis. We have found no differences in either direction between the groups. There was no difference in the surface CD89 expression in either monocytes or neutrophils but we did find a trend towards reduced receptor expression in patients with PIgAN and cirrhosis but this did not achieve statistical significance. When examining the percentage of CD89 positive cells, it appears that the values for percentage receptor positivity in monocytes in all groups show a wide scatter about the mean with no significant difference between the groups. This suggests that in any one group, the monocyte population is quite heterogeneous with regards to the presence of CD89 receptors on the cell surface. Conversely a high percentage of CD89 positivity existed in the neutrophils of normal controls, patients with GN and HIgAN with only a few individuals in each group showing lower levels of receptor positivity. The patients with cirrhosis and to lesser extent patients with primary IgAN on the whole had a lower percentage of CD89 positive neutrophils. These findings did not achieve statistical significance which may be explained by the small numbers of patients especially in the cirrhosis group.

Given the standardised experimental procedure, it is unlikely that the lack of significant differences between groups in CD89 expression is due to the presence of different variables affecting the results during the course of our experiments. The standardised protocol was applied consistently to every set of experimental sample and conditions were optimised for minimal leukocyte activation. Furthermore, the populations of lymphocytes, monocytes and polymorph nuclear cells were live-gated according to FSc and SSc characteristics and the accuracy of these gates confirmed by back-gating using CD14 and CD45 staining. The conjugated antibodies used for cell staining were all standard commercial ones with high levels of specificity for their receptors. For accurate quantitation the amount of antibodies used was of saturating level. The appropriate amount was determined empirically prior to these experiments. The flow cytometer was calibrated daily for fluorescence and light scatter with

microbead standards (Dako FluoroSpheres, Dako Ltd). During the time course of the study no significant change in calibration was seen.

The other aspect of the methodology which may account for the discrepancies in the findings by different researchers is the antibody technique used to stain the cells under examination. Kashem et al (1996) had used indirect immunofluorescence (IF) in their experiments which may cause cross-linking by the secondary antibody in the IF assay. This in turn could cause clustering of the receptor resulting in falsely elevated fluorescence intensity. Grossetete et al 1998 used direct antibody binding to the receptor to avoid this problem. In fact they did report a difference in their results depending on which technique they utilized. A direct assay with a fluorochrome labelled anti-CD89 antibody showed significantly decreased fluorescence intensity (FI) whereas repeating the same experiment with an indirect assay yielded a slightly increased FI value in IgAN. However, having used the same direct fluorochrome conjugated anti-CD89 monoclonal antibody, we have not found statistically significant decrease in surface expression of CD89 in circulating monocytes and neutrophils.

We did find some evidence of cell activation from the data assessing side scatter in granulocytes. The only groups that were statistically different in terms of granularity were those patients with cirrhosis without renal involvement. It is unlikely that this is due to samples from these patients being handled differently during the course of the study as no statistically significant correlation was found between the neutrophil CD89 expression and the side scatter for the samples. It is most likely that these patients' cells were activated *in vivo*. This finding however does not explain the lack of difference in the receptor expression in neutrophils in the different patient groups. Previous studies of CD89 receptor expression on monocyte cell lines (U937) suggest that cells activated with PMA show increased intensity of surface staining up to 3-4 fold, consistent with activation induced by IgA binding up-regulation. One explanation for finding the reverse in our cirrhotic patients may be the occupation of these receptors by IgA immune complexes which are found in abundance in the circulation of these patients, thus decreasing monoclonal antibody binding and hence staining of the cell surface receptors. In our results however, we did not find a correlation between total serum IgA levels in patients and fluorescence intensity.

In summary, our experiments did not show statistically significant over expression or under expression of CD89 in neutrophils and monocytes in our study groups when compared with normal and other relevant controls. These findings effectively negate the hypothesis that the differences in the serum IgA levels in patients with IgAN and liver disease are due to altered expression of and clearance via the monocyte and neutrophil CD89 receptor. Our study did show a trend towards reduced CD89 expression on neutrophils in primary IgAN and cirrhosis as well as lower percentage of CD89 positive neutrophils in these two patient groups, but these findings did not achieve statistical significance. This may be due to small numbers of patients in the hepatic groups.

The differences in the results between the different studies presented in this thesis may ultimately be due to the study of vastly different populations of patients with different genetic characteristics. IgA nephropathy in itself is a heterogeneous disease and this per se renders extrapolation from one study performed in one population to another with vastly different racial and genetic backgrounds problematic. However, based on our findings, it is reasonable to conclude that CD89 expression does not play a key role in determining the levels of serum IgA in IgAN and hepatic cirrhosis and the predisposition to tissue IgA deposition. Other factors such as the ASGPR function and the state of immune activation and over-production of IgA are a more likely explanation of the finding of raised IgA levels in these conditions. Our studies of IgA production in these patients are presented in the following chapter (7).

# Chapter 7: IgA Production in IgA nephropathies

## 7.1 Human IgA production

Despite the marked predominance of mucosal IgA production, appreciable quantities of IgA are also made in systemic immune sites, most notably the bone marrow. In health this IgA is nearly entirely monomeric IgA1, which is secreted into the circulation (Kerr 1990). There have been reports of increased serum IgA levels in the primary IgA nephropathy literature (d'Amico 1988). The ratio of polymeric to monomeric IgA in the circulation although within the normal range, is reported as increased when compared to normal subjects (Layward et al 1992; Ots et al 1999). Both patients with cirrhosis and hepatic IgAN are said to have elevated circulating IgA levels (Feehally 1988; Pouria and Feehally 2000). Whilst in normal individuals and those with primary IgAN the circulating IgA is predominantly of the IgA1 sub-type, in cirrhosis both IgA1 and IgA2 levels are elevated, the latter being the more markedly raised.

In this chapter the data from studies assessing the characteristics of serum IgA and its production, as set out in the aims of the thesis are presented. These experiments were performed to address the following points:

1. In order to assess any differences in IgA production between the patient and control groups serum IgG, IgA, and IgA sub-class (IgA1 and 2) levels in both primary and secondary IgAN and their respective controls were measured as per protocols described in chapter 3.
2. We also measured total IgA, IgG and IgM levels in the supernatant produced by peripheral blood mononuclear cells in stimulated and unstimulated cultures to find if there were any differences in immunoglobulin production between the groups in vitro.
3. We measured the levels of IgA monomer and polymer in the serum of the different groups by size exclusion chromatography looking for differences in the ratio of polymeric and monomeric IgA in the different patient and control groups.

## 7.2 Results

### 7.2.1 Serum immunoglobulin levels

*7.2.1.1 Serum total IgA*: Total serum IgA levels were measured in 23 normal controls, 15 patients with PIgAN, 10 patients with other glomerulonephritides, 6 patients with hepatic IgAN and 11 patients with alcoholic cirrhosis. Total IgA levels obtained by ELISA in normal controls were between 1.7 and 7.5 mg/ml with a mean and standard deviation of 4.4±1.8. Patients with IgA nephropathy (both primary and hepatic) had significantly elevated total IgA levels as expected. The range for the former was between a minimum of 3.9 and a maximum of 15.4 mg/ml and in the latter between 5 and 16.6 mg/ml. Patients with cirrhosis with no renal disease had the highest IgA levels of all the groups as previously reported in the literature, with a range of 4-31.7 mg/ml and a mean of 17.7±7.9 mg/ml. Unexpectedly we found that even patients with non-IgA glomerulonephritis had a modest but statistically significant elevation in their total serum IgA levels with a range of 3.6-12.5 and a mean of 6.6±2.7mg/ml. These results are summarised in **Table 7.1** and depicted in the graph in **figure 7.1.**

The serum IgA levels measured using ELISA in our hands in the laboratory were higher than the range quoted from our hospital laboratories. We found that on average the levels were greater by 2-4 fold. We speculate that this arose as a result of a commercial RID kit being used by the hospital laboratories. The higher sensitivity of the ELISA probably explains this difference. Nevertheless as demonstrated by the dot-plot graph in **Figure 7.2,** there is a good correlation between the laboratory values measured by us and using the hospital immunology RID kit.

*7.2.1.2 Serum IgA1*: Serum IgA1 samples were measured by ELISA as per methods outlined in chapter 3. Samples were analysed from the same patient groups as before. The normal range for serum IgA1 was between 0.4 to 5.8 mg/ml with a mean of 3.1 ± 1.6. IgA1 levels were significantly elevated in all the patient and control groups except for HIgAN where the increased mean IgA1 value did not reach statistical significance due to small sample size. These changes were particularly marked in patients with cirrhosis with no renal disease although we did find a very wide scatter around the mean. A summary of these results is shown in **Figure 7.3** and **Table 7.2.**

| | Normal | PIgAN | GN | HIgAN | Cirrhosis |
|---|---|---|---|---|---|
| **Number** | 23 | 15 | 10 | 6 | 11 |
| **Mean** | 4.32 | 8.10 | 6.57 | 10.37 | 17.73 |
| **SD** | 1.75 | 2.98 | 2.66 | 4.23 | 7.86 |
| **SEM** | 0.36 | 0.77 | 0.84 | 1.73 | 2.37 |
| **P value** | N/A | 0.0001* | 0.01* | 0.001* | 0.0001* |

**Table 7.1** Statistical analyses of values obtained for total serum IgA and the differences between different patient groups. Serum total IgA levels were significantly elevated in all groups as compared with normal. The data were analysed using a Mann Whitney U test with confidence intervals of greater than 95%.

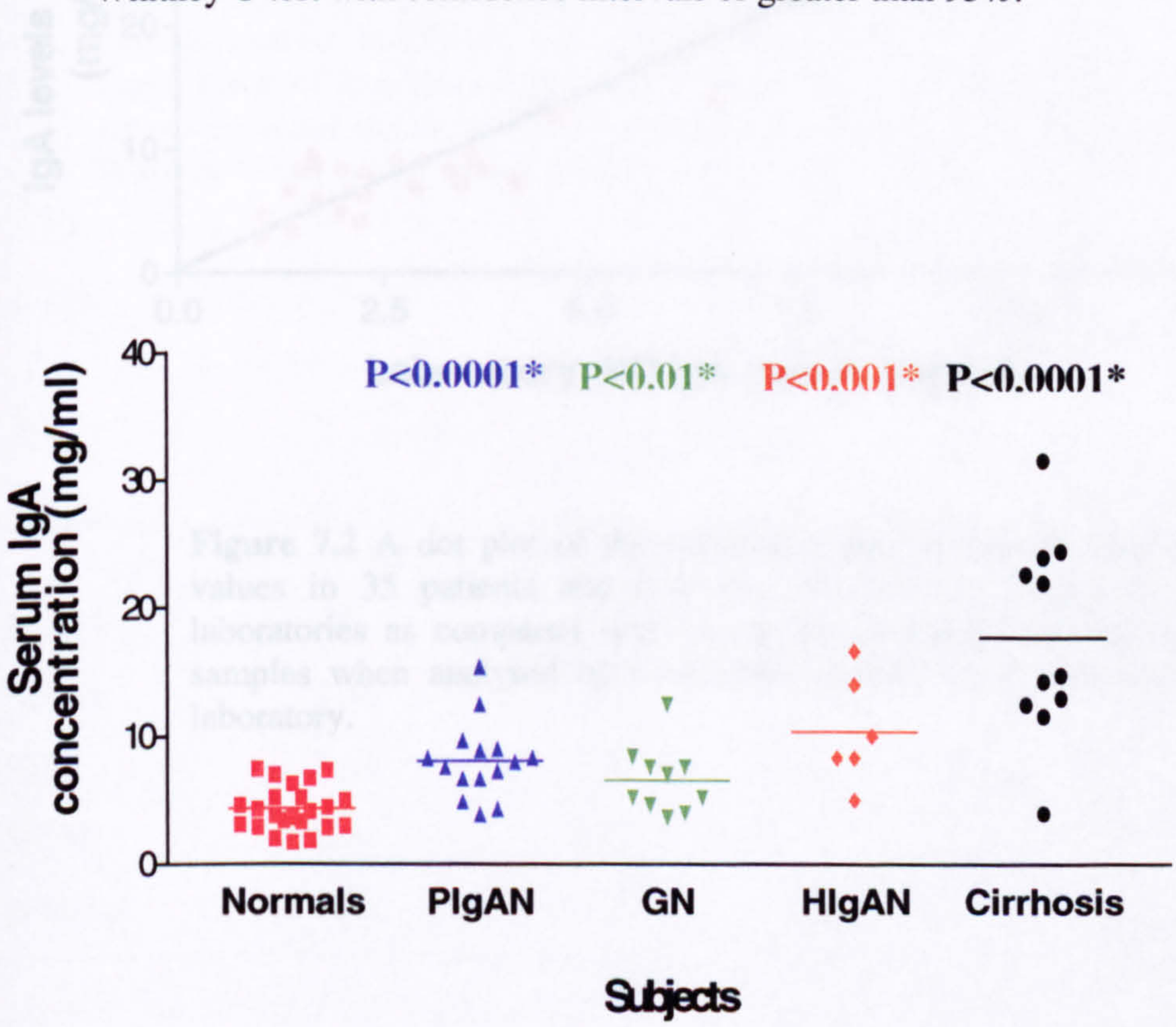



**Figure 7.1** A graph comparing total serum IgA levels in the different patient groups, showing statistically significant elevation in all patient and control groups as compared with the normal controls.

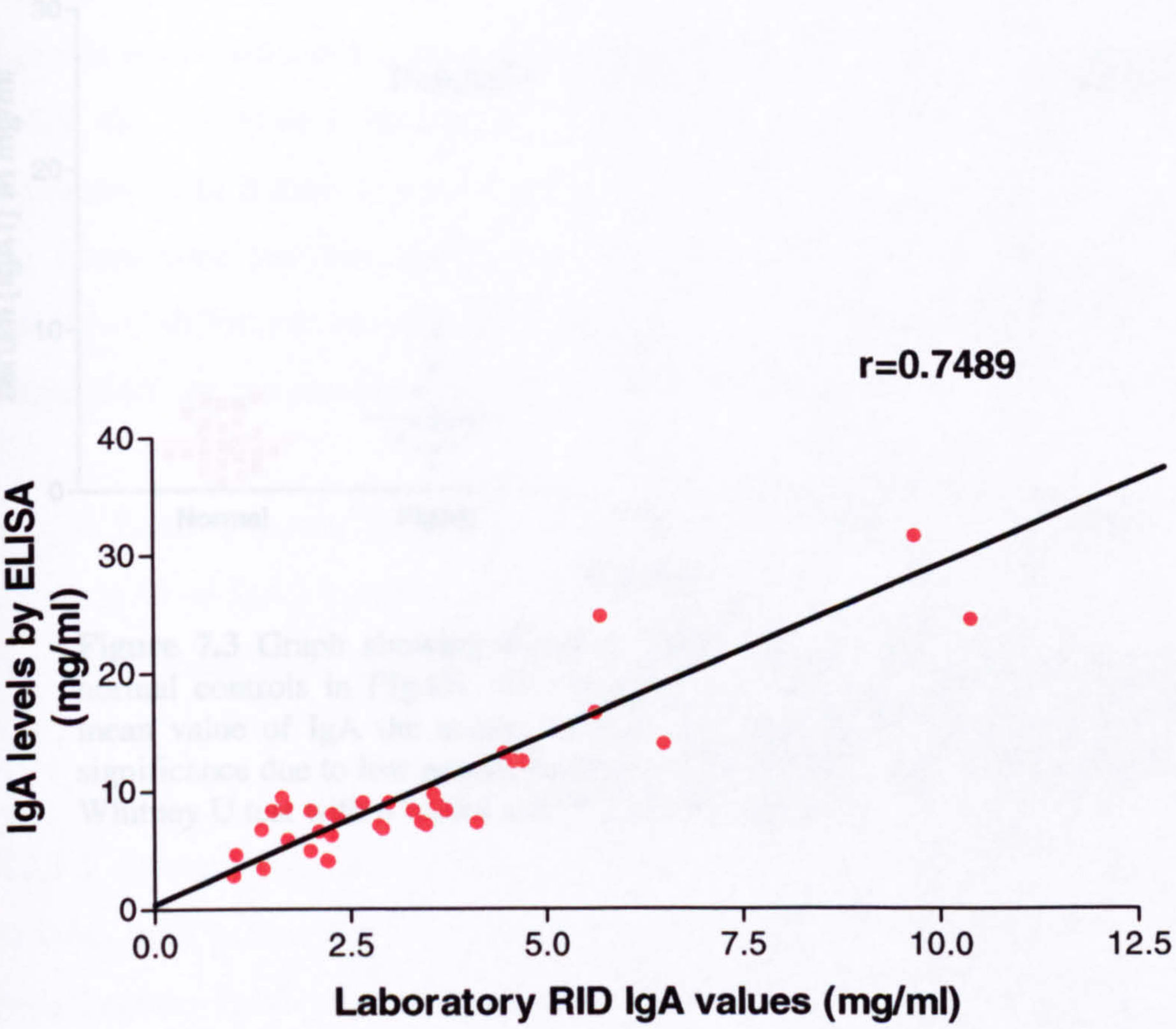



**Figure 7.2** A dot plot of the correlation between serum total IgA values in 35 patients and controls, obtained by ELISA in our laboratories as compared with the levels obtained from the same samples when analysed by a commercial RID kit in the hospital laboratory.

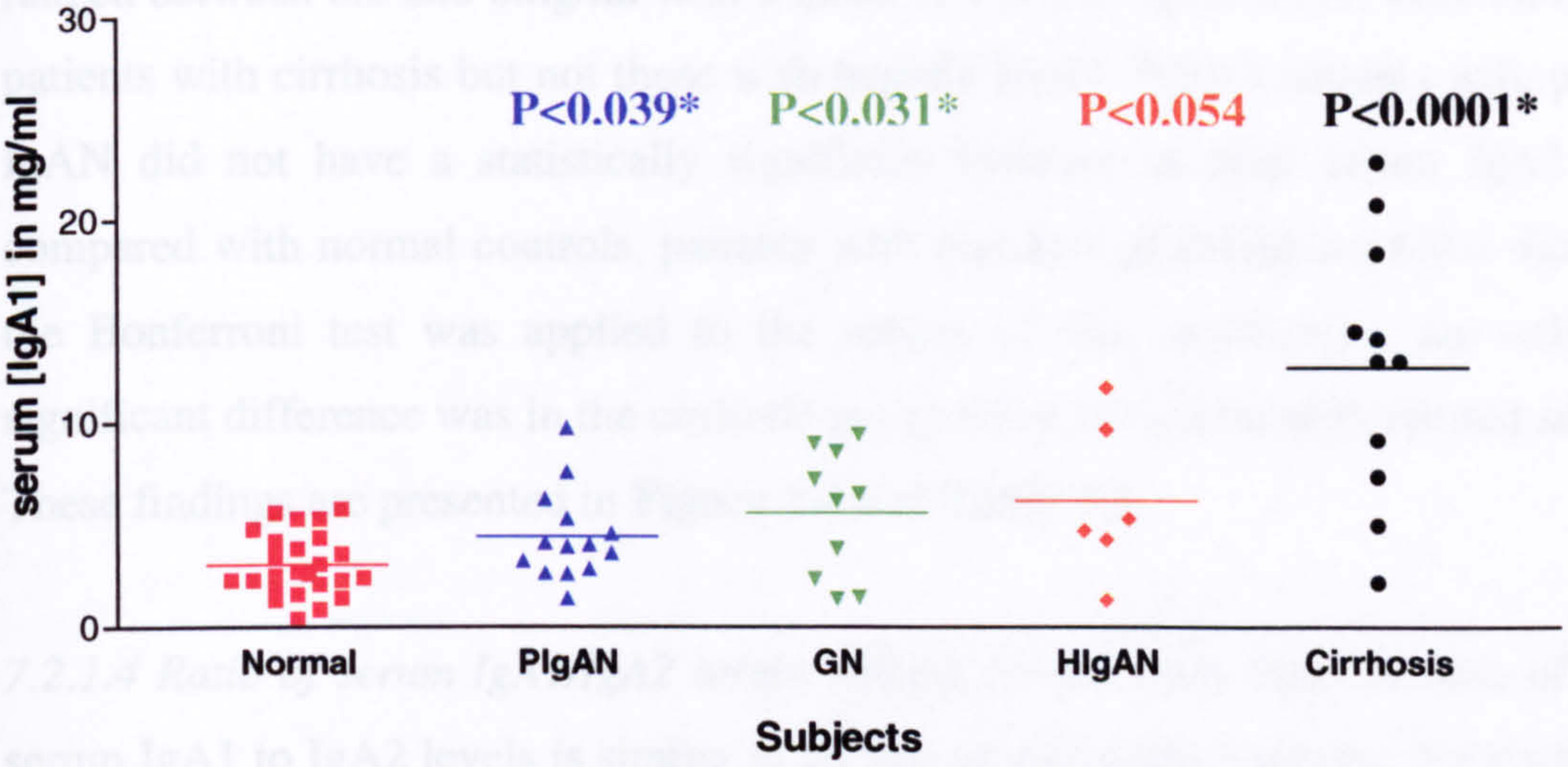



**Figure 7.3** Graph showing elevated serum IgA1 in patients as opposed to normal controls in PIgAN, GN, HIgAN and Cirrhosis. Despite the elevated mean value of IgA the results in the HIgAN group did not reach statistical significance due to low patient numbers. Analyses were performed using Mann Whitney U test with p values < 0.05 deemed significant.

| | Normal | PIgAN | GN | HIgAN | Cirrhosis |
|---|---|---|---|---|---|
| **Number** | 25 | 14 | 10 | 6 | 11 |
| **Mean** | 3.10 | 4.43 | 5.61 | 6.20 | 12.81 |
| **SD.** | 1.53 | 2.20 | 3.12 | 3.83 | 6.47 |
| **SEM** | 0.31 | 0.59 | 0.99 | 1.57 | 1.95 |
| **p value** | N/A | 0.039* | 0.031* | 0.054 | 0.0001* |

**Table 7.2** Table showing differences in serum IgA1 levels between different patient groups. Patients with primary and not hepatic IgA nephropathy have statistically significant elevated levels of circulating IgA1, as do patients with other glomerulonephritis and cirrhotics with no renal disease.

*7.2.1.3 Serum IgA2*: Serum IgA2 levels were measured by ELISA in the same patient and control groups as per the protocol described in chapter 3. The normal levels ranged between 0.2 and 8mg/ml with a mean of 1.9 ± 2. IgA2 levels were elevated in patients with cirrhosis but not those with hepatic IgAN. Whilst patients with primary IgAN did not have a statistically significant increase in their serum IgA2 levels compared with normal controls, patients with non-IgA glomerulonephritis did. Once the Bonferroni test was applied to the results of this experiment, the only truly significant difference was in the cirrhotic group when compared with normal subjects. These findings are presented in **Figure 7.4** and **Table 7.3.**

*7.2.1.4 Ratio of serum IgA1/IgA2 levels:* These results show that the ratio of paired serum IgA1 to IgA2 levels is similar in all patient and control groups. No statistically significant differences were found using the Mann Whitney U test as shown in **figure 7.5.**

*7.2.1.5 Serum IgG*: Serum IgG levels were measured in 26 normal controls, 16 patients with primary IgAN, 10 patients with other glomerulonephritides, 5 patients with hepatic IgAN and 11 patients with alcoholic cirrhosis with no glomerular disease. The results show that serum IgG levels from normal serum had a range between 7.2 to 33 mg/ml with a mean of 18 mg/ml. Only patients with primary IgAN and cirrhosis when compared to normal controls appear to have a statistically significant increase in their serum IgG levels whereas those with other glomerulonephritides and secondary IgAN do not when the Mann Whitney U test is applied alone. The difference between PIgAN and GN is also statistically significant suggesting that the raised IgG levels are not related to renal disease per se but specific to PIgAN. We conclude that the rise in IgG levels may be a result of inflammation per se and not necessarily a feature of either liver or renal disease since patients with hepatic IgAN seem to have levels within the normal range. **Figure 7.6** and **Table 7.4** show the details of the results of this experiment.

## 7.2.2 Polymeric and monomeric IgA production

Serum IgA1 purified by jacalin affinity chromatography was used for this study as described in the chapter on Methods and Materials. Samples were used from the

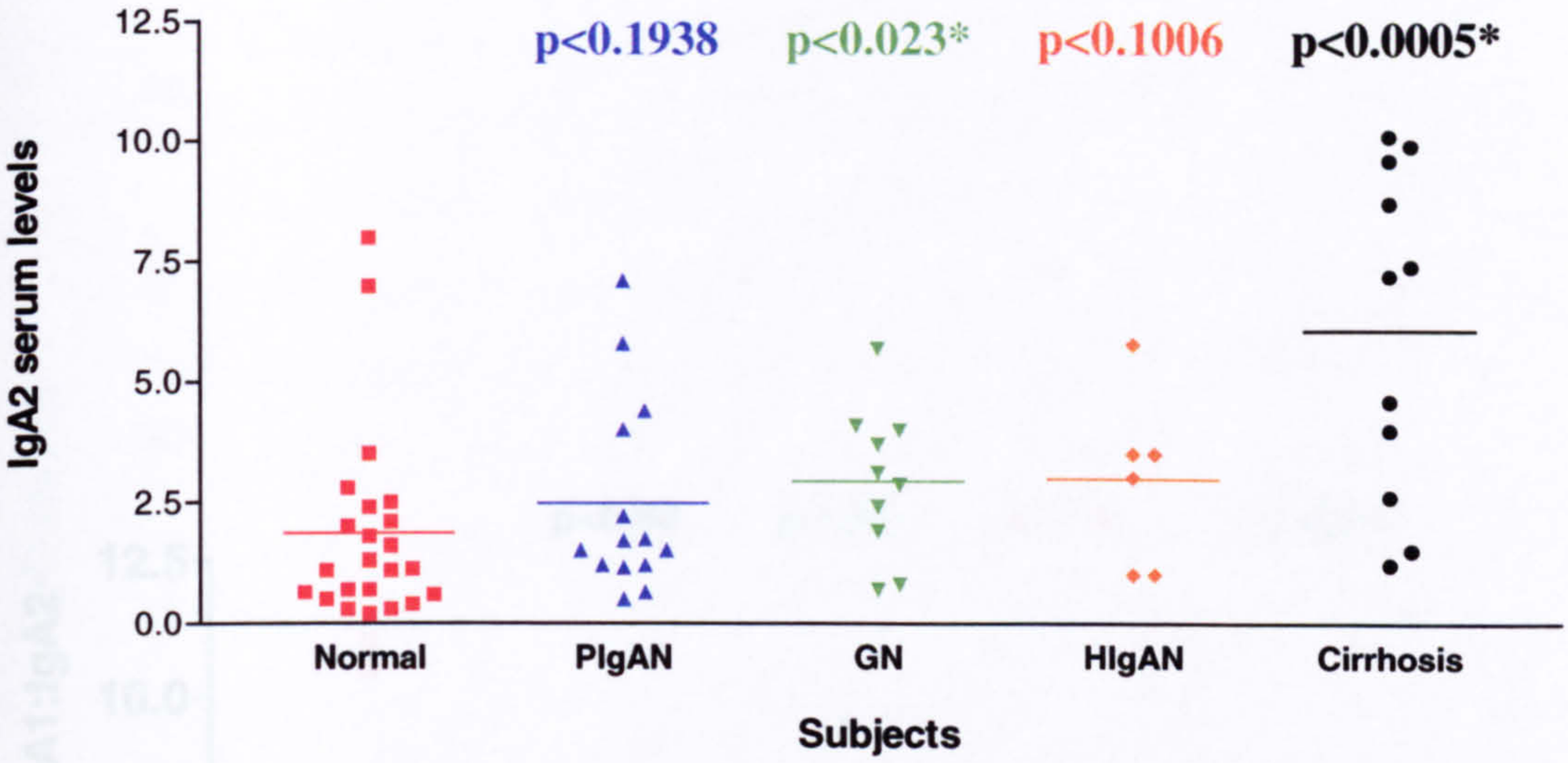



**Figure 7.4** Graph showing serum IgA2 levels in different patients. IgA2 levels were significantly elevated in patients with cirrhosis and patients with non-IgA glomerulonephritis and not in primary or hepatic IgANs. Once the Bonferroni test was applied to the results, the only significant result was in the difference found between patients with cirrhosis and normal controls.

| | Normal | PIgAN | GN | HIgAN | Cirrhosis |
|---|---|---|---|---|---|
| **Number** | 23 | 14 | 10 | 6 | 11 |
| **Mean** | 1.86 | 2.47 | 2.93 | 2.97 | 6.07 |
| **Std. Dev.** | 2.00 | 2.04 | 1.55 | 1.81 | 3.41 |
| **Std. Error** | 0.42 | 0.54 | 0.49 | 0.74 | 1.03 |
| **p value** | N/A | 0.194 | 0.023* | 0.101 | 0.0005* |

**Table 7.3** Table summarising the statistical analyses on results from serum IgA2 levels and the differences found between different patient groups. (N: normal controls, P: PIgAN, G: other glomerulonephritides, H: hepatic IgAN, C: cirrhosis, * denotes statistical significance by the MWU test)

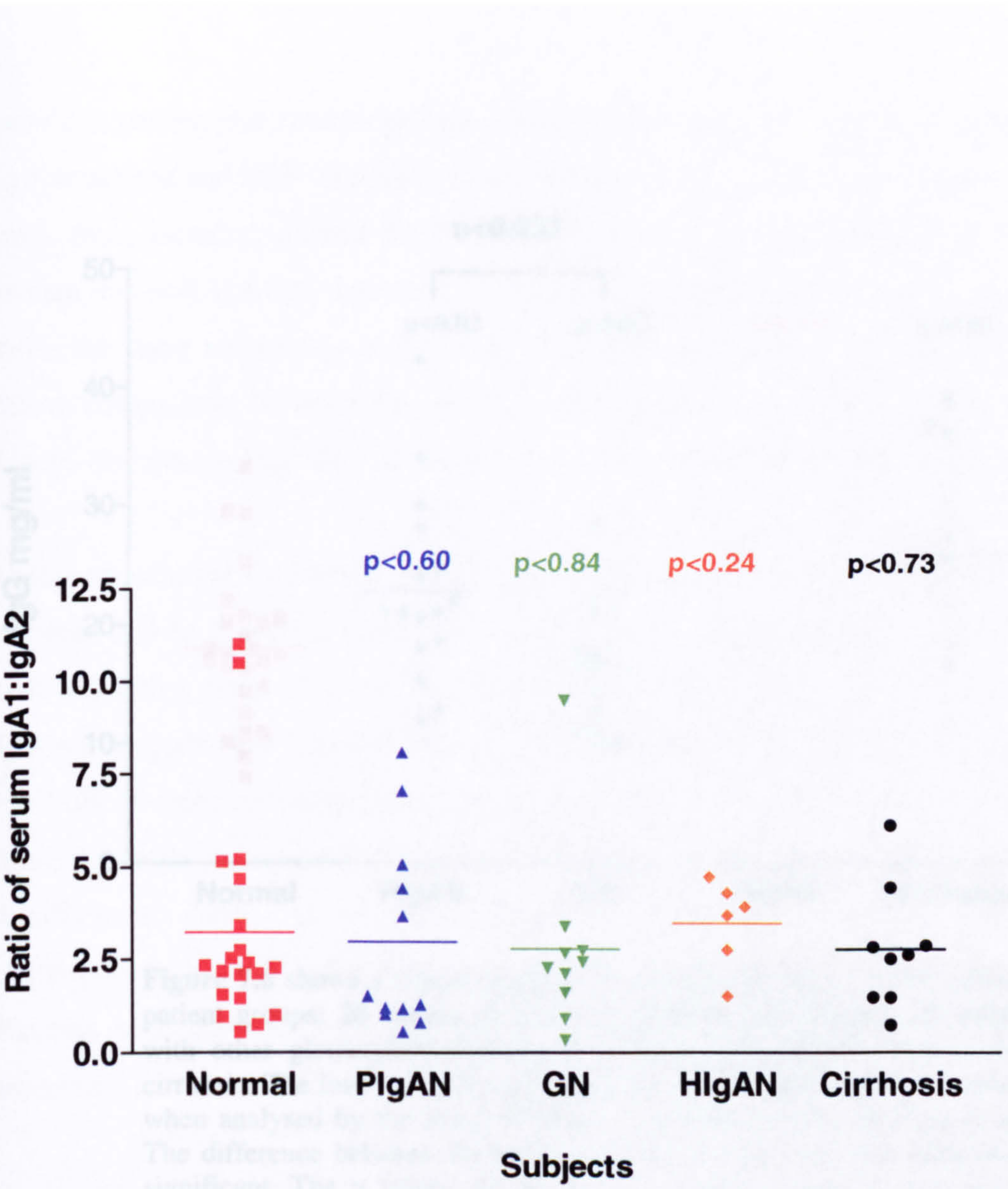



**Figure 7.5** Graph depicting the ratio of IgA1 to IgA2 in serum from the different patient and control groups. The horizontal bar is the mean value for each group. There was no statistically significant difference between the means in any of the patient groups when compared with normal controls. The data were analysed by a Mann Whitney U test

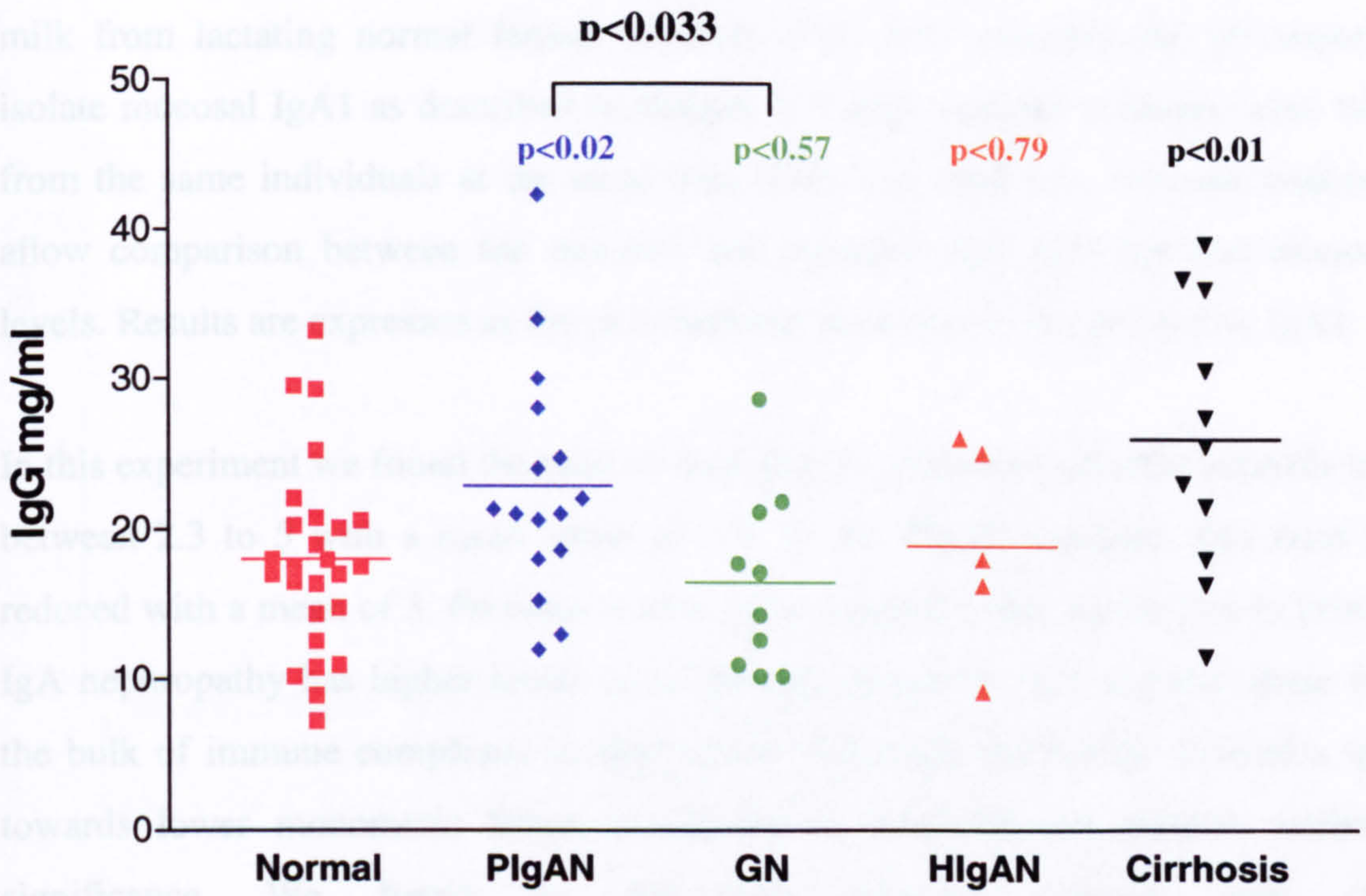



**Figure 7.6** shows a scatter graph of the serum IgG levels in the different patient groups: 26 normal controls, 16 patients with PIgAN, 10 patients with other glomerulonephritides, 5 patients with HIgAN, and 11 with cirrhosis. The level of IgG production was significantly elevated cirrhosis when analysed by the Mann Whitney U followed by the Bonferroni test. The difference between the PIgAN and GN groups was also statistically significant. The p values shown are for patients compared with normal controls.

| | Normal | PIgAN | GN | HIgAN | Cirrhosis |
|---|---|---|---|---|---|
| **Number** | 26 | 16 | 10 | 5 | 11 |
| **Mean** | 18 | 23 | 16 | 19 | 26 |
| **SD** | 6.3 | 7.8 | 6 | 6.9 | 9 |
| **SEM** | 1.2 | 2 | 1.9 | 3.1 | 2.7 |
| **p value** | N/A | 0.02* | 0.57 | 0.79 | 0.01* |

**Table 7.4** Statistical analyses of the serum IgG levels: Tables showing statistical differences in serum IgG levels between different patient groups. (N: normal controls, P: PIgAN, GN: other glomerulonephritides, H: hepatic IgAN, C: cirrhosis, * denotes statistically significant p value with 95% confidence intervals using Mann Whitney U test)

previous patient and control groups. Additionally, serum samples from patients with IgA myeloma and HSP secondary to myeloma were also used. Two samples of breast milk from lactating normal female controls were also collected and processed to isolate mucosal IgA1 as described in chapter 3. Paired samples of serum were taken from the same individuals at the same time. This was used as a mucosal control to allow comparison between the mucosal and systemic IgA polymer and monomer levels. Results are expressed as the ratio between monomeric and polymeric IgA1.

In this experiment we found the ratio of monomer to polymer in normal controls to be between 2.3 to 5 with a mean value of 3.4. In the PIgAN samples, this ratio was reduced with a mean of 3. Previous studies have suggested that serum IgA in primary IgA nephropathy has higher levels of circulating polymeric IgA and that these form the bulk of immune complexes in their blood. Although our results showed a trend towards lower monomeric forms in the serum, they did not achieve statistical significance. We found no difference between patients with other glomerulonephritides and the normal and the same holds true for hepatic IgAN. Patients with cirrhosis however had a much lower monomer/polymer ratio with a mean of 2.2. This was highly significant with $p<0.0011$.

The patients with HSP and myeloma had normal monomeric/polymeric IgA1 ratios where as patients with IgA multiple myeloma had very high levels of polymeric IgA1 and therefore a much reduced monomeric/polymeric ratio of 0.96. The breast milk contained very low levels of monomer, with one sample having an m/p ratio of 0.13 and the other 0.5 (mean=0.34). This finding is to be expected given that the majority of secretory IgA is known to be polymeric. The results of these studies are shown in **Table 7.5** and **Figure 7.7.**

### 7.2.3 Supernatant immunoglobulin production

In our study, no differences were found between the patient groups in their ability to produce immunoglobulins of any of the IgA, IgG or IgM classes. Basal IgA production levels were virtually similar in all groups (**Figure 7.8**). Whilst we found that stimulation with pokeweed mitogen did increase the amount of IgA found in the supernatant of the cultured PBMCs, there was again no significant difference between the groups (**Figure 7.9**).

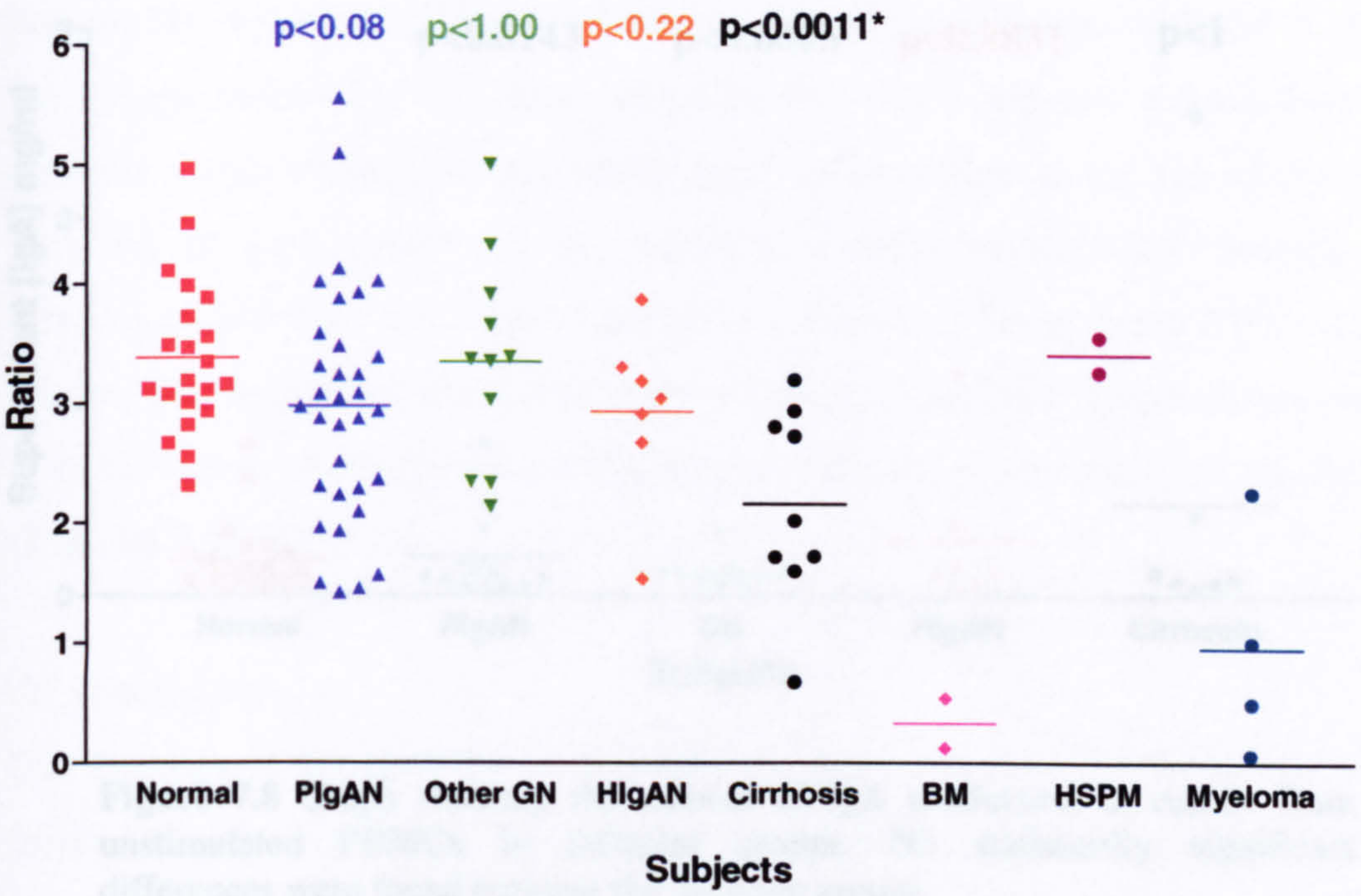



**Figure 7.7** Graph showing the ratio of monomeric to polymeric IgA in different patients. In addition to the 5 patient and control groups 2 samples of breast milk (BM) from 2 lactating normal females (mucosal IgA), 2 samples of serum from patients with Henoch-Schönlein Purpura secondary to an IgA Myeloma (HSPM) and 4 samples from patients with different IgA myelomas were used. (BM: breast milk; HSPM: Henoch-Schonlein Purpura secondary to Myeloma).

| | Normal | PIgAN | GN | HIgAN | Cirrhosis |
|---|---|---|---|---|---|
| **Number** | 21 | 33 | 11 | 7 | 9 |
| **Mean** | 3.40 | 2.98 | 3.35 | 2.93 | 2.16 |
| **Std. Dev.** | 0.65 | 0.99 | 0.88 | 0.72 | 0.82 |
| **Std. Error** | 0.14 | 0.17 | 0.27 | 0.27 | 0.27 |
| **p value** | N/A | 0.08 | 1 | 0.22 | 0.0011* |

**Table 7.5** shows the analyses on the ratio of monomeric to polymeric IgA1 by size exclusion chromatography in the different patient groups. Only patients with cirrhosis have statistically significant reduction in the ratio of monomeric to polymeric IgA in their circulation. Although the ratio was reduced in patients with both primary and hepatic IgAN, this did not attain statistical significance by the Mann Whitney U test. Statistical analyses were not carried out on the myeloma patients and the 2 samples of breast milk due to the small numbers of cases.

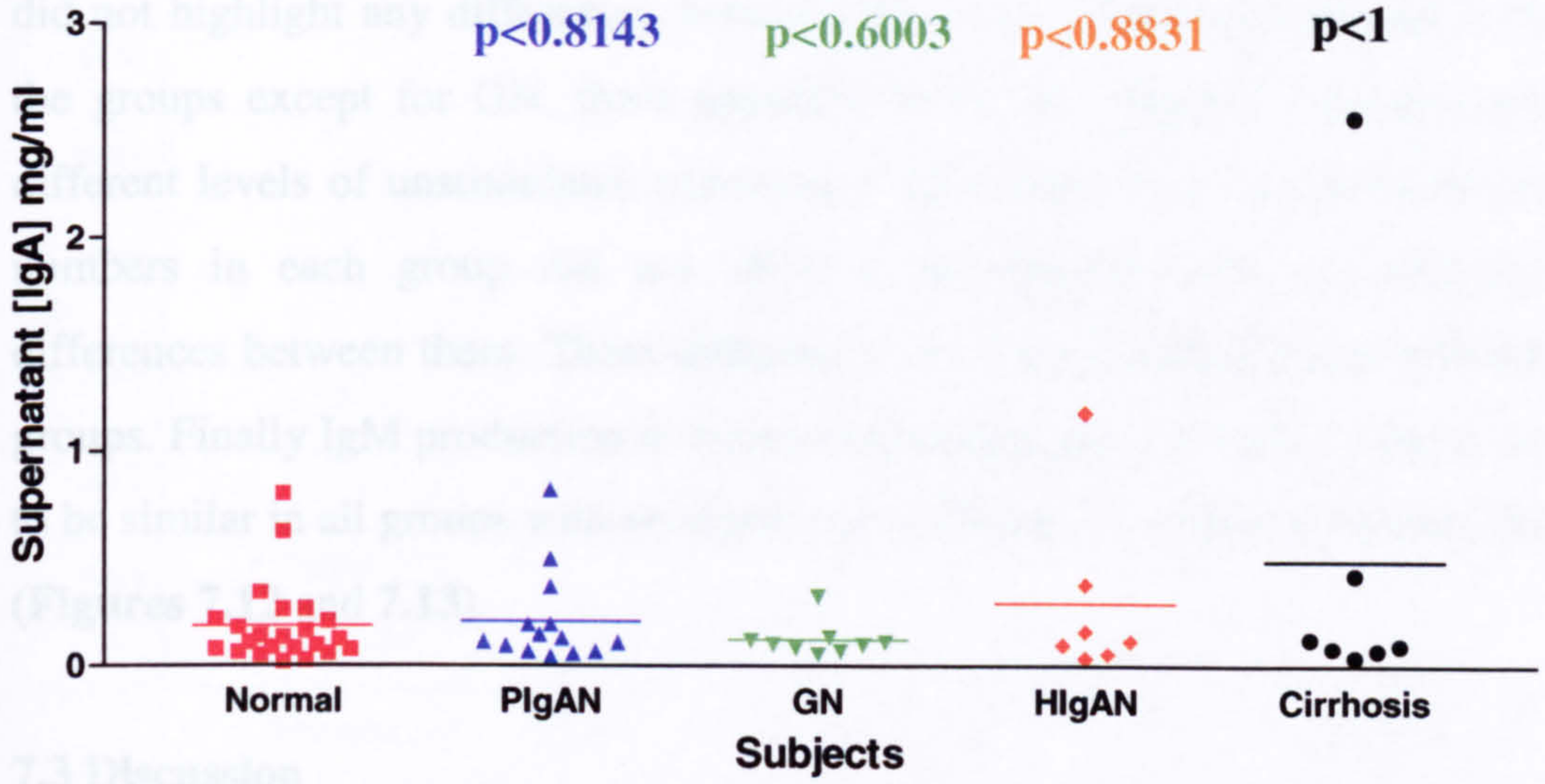



**Figure 7.8** Graph showing the amount of IgA production in culture from unstimulated PBMCs in different groups. No statistically significant differences were found between the different groups.

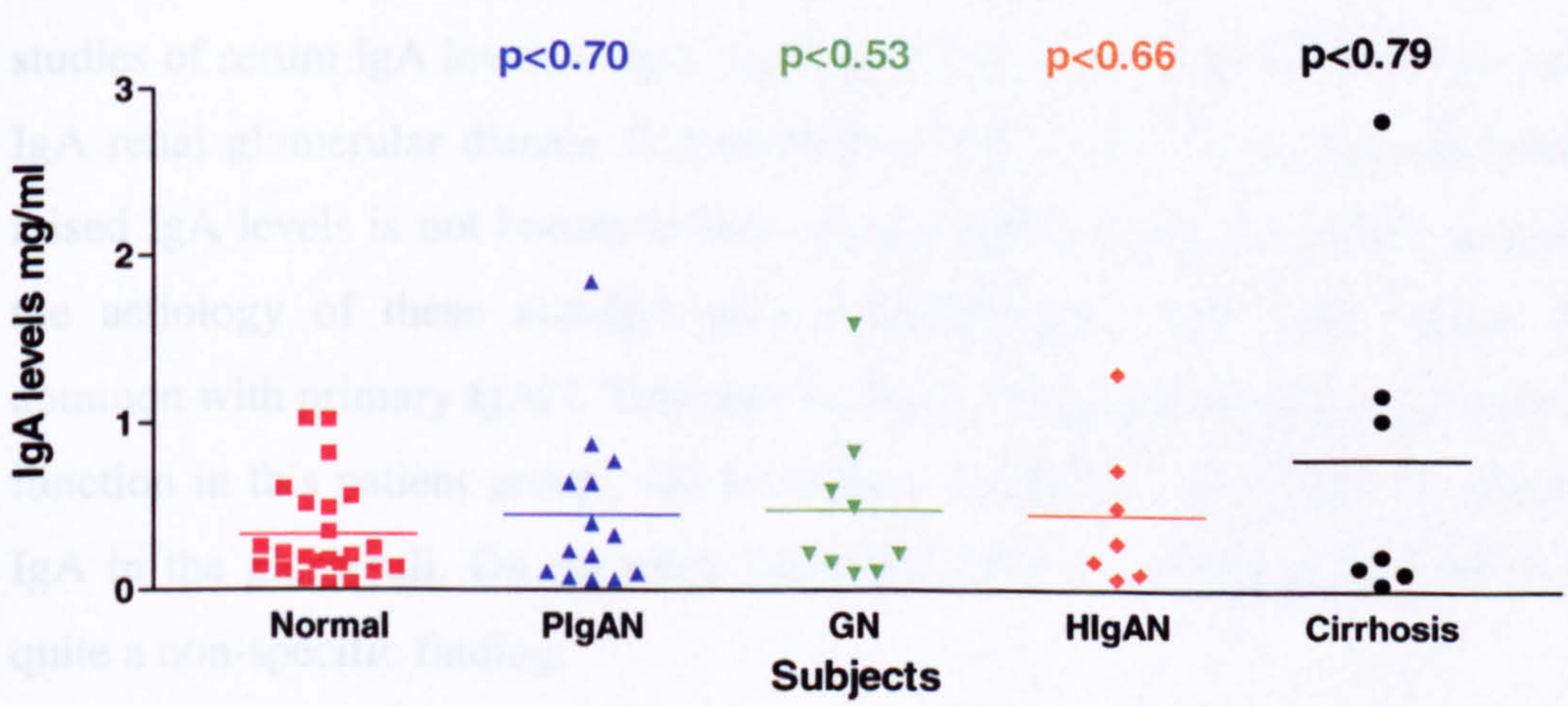



**Figure 7.9** Graph showing the level of IgA production from PBMCs stimulated in culture with pokeweed mitogen in the different patient groups. No statistically significant differences were found between the different groups.

Similarly the results of experiments looking at basal and stimulated IgG production did not highlight any differences between the groups (**Figures 7.10** and **7.11**). In all the groups except for GN, there appeared to be two separate subpopulations with different levels of unstimulated supernatant IgG production, but the relatively small numbers in each group did not allow a meaningful statistical analysis of the differences between them. These differences were also found in the PWM stimulated groups. Finally IgM production in both unstimulated and stimulated culture was found to be similar in all groups with no significant differences observed between the groups (**Figures 7.12** and **7.13**).

## 7.3 Discussion

Our findings in these series of experiments support previous reports that total IgA levels are generally elevated in patients with cirrhosis and in primary IgAN. We also found a significant increase in total serum IgA levels in patients with hepatic IgAN. However the finding that IgA levels are elevated in patients with other types of glomerulonephritis was surprising and may suggest mechanisms involved in serum IgA elevation which are not specific to IgA nephropathies. Some of the previous studies of serum IgA levels in IgA nephropathy have not used controls that have non-IgA renal glomerular disease (Layward et al 1993). The underlying mechanism for raised IgA levels is not known in this group of patients and one might speculate that the aetiology of these non-IgA glomerulonephritides may have factors that are common with primary IgAN. This may be due to irregularities in the mucosal immune function in this patient group, which contrary to PIgAN do not lead to deposition of IgA in the glomeruli. On the other hand the presence of higher IgA levels may be quite a non-specific finding.

In the analysis of the sub-classes of IgA, all the different patient and control groups had elevated IgA1 levels as compared with normal controls. This was not expected in patients with non-IgA glomerulonephritis. Interestingly these patients showed significantly elevated IgA2 levels as compared with normals even when these levels were found to be normal in patients with primary and hepatic IgAN. Patients with cirrhosis also had highly elevated IgA2 levels. This has been previously reported in the literature (Kutteh et al 1982).

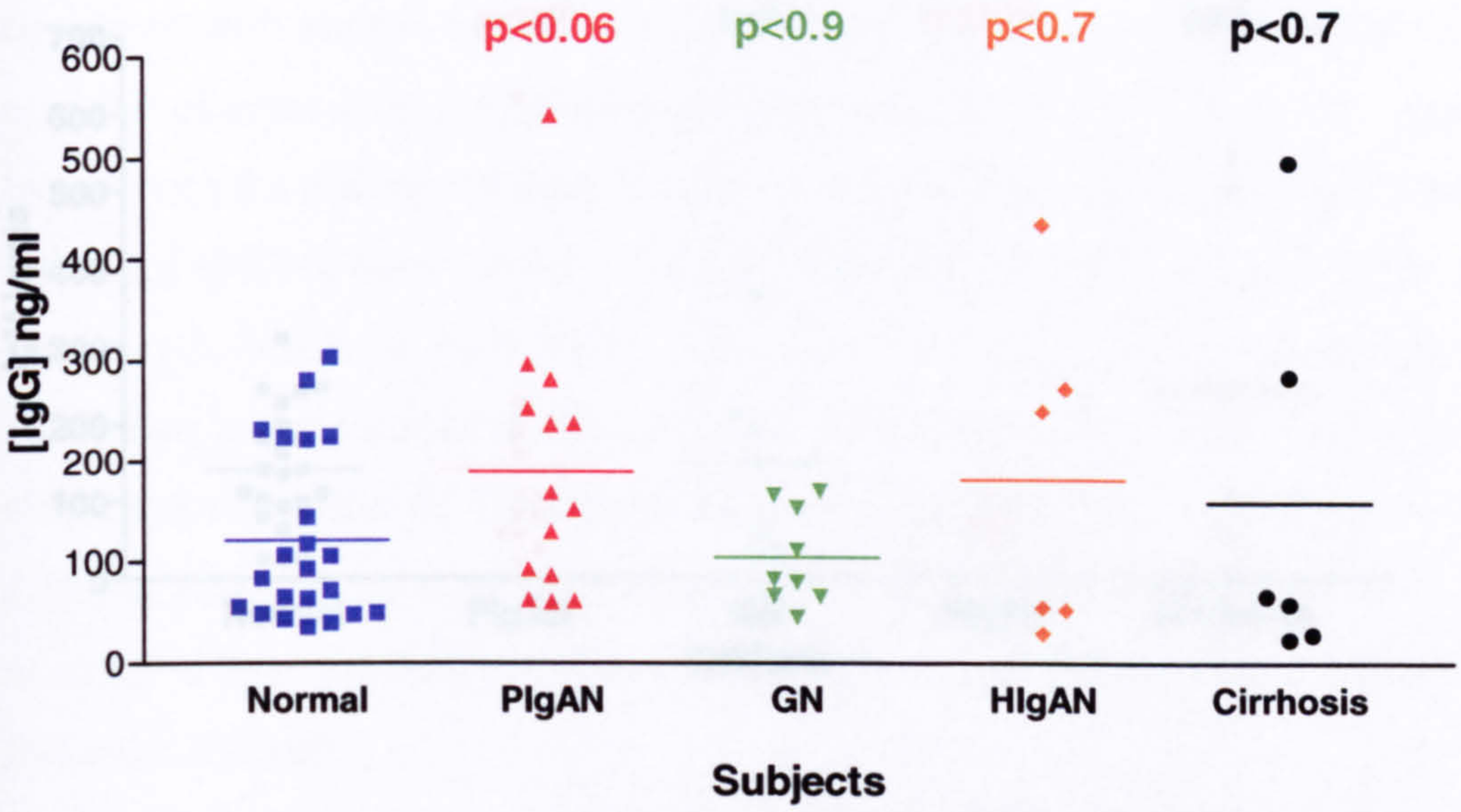



**Figure 7.10** IgG levels produced by PBMCs in unstimulated culture medium showing no statistical differences between the patient and control groups. There was a bimodal distribution of IgG levels within each individual group suggesting the presence of sub-populations with different IgG producing capacity.

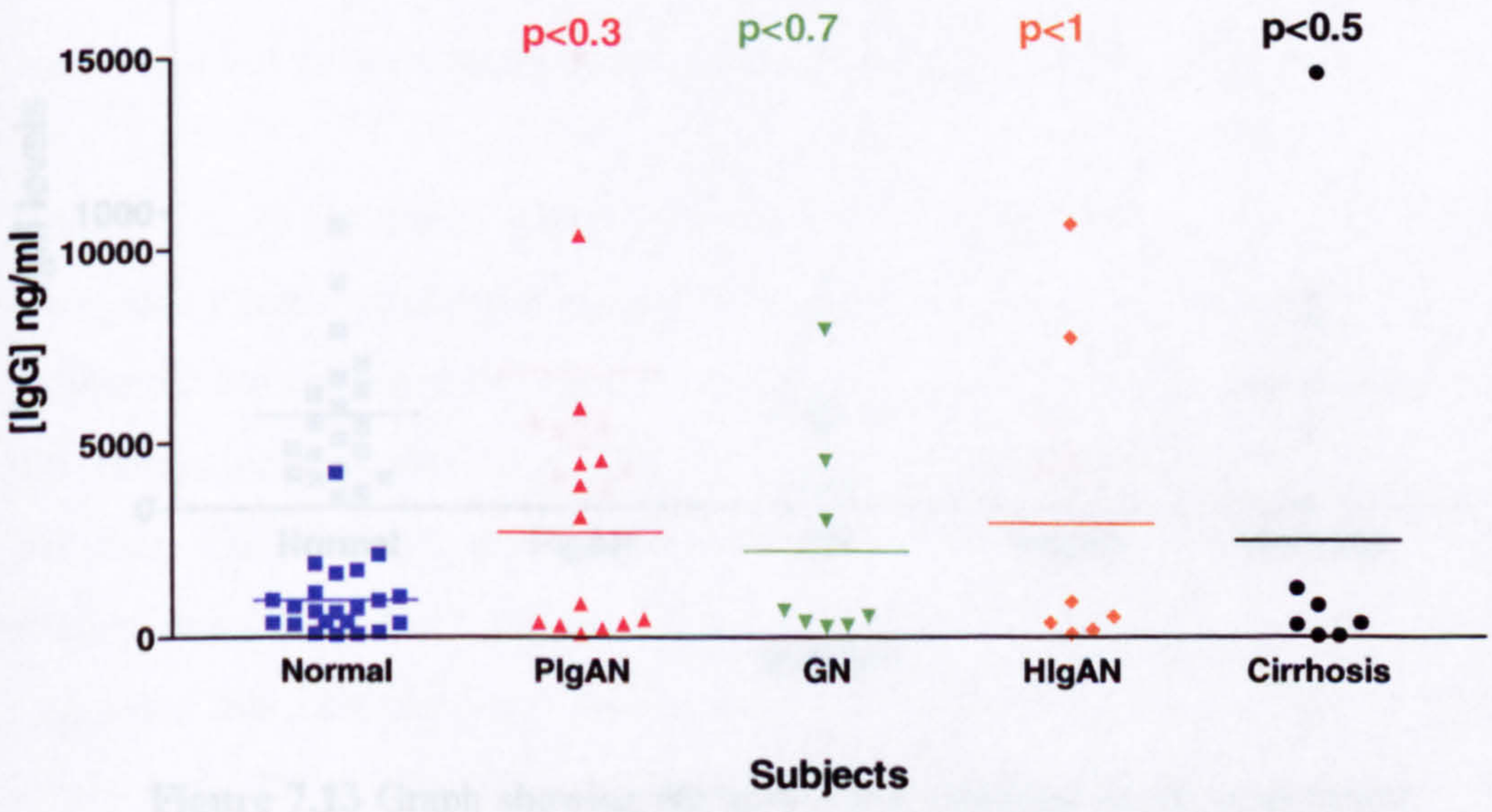



**Figure 7.11** IgG levels in supernatant produced by cultured PBMCs stimulated with PWM. There was no significant difference in the results between the different groups. The same bi-modal distribution as that found in basal IgG secretion is visible in stimulated IgG production, with the exception of the results from the normal controls.

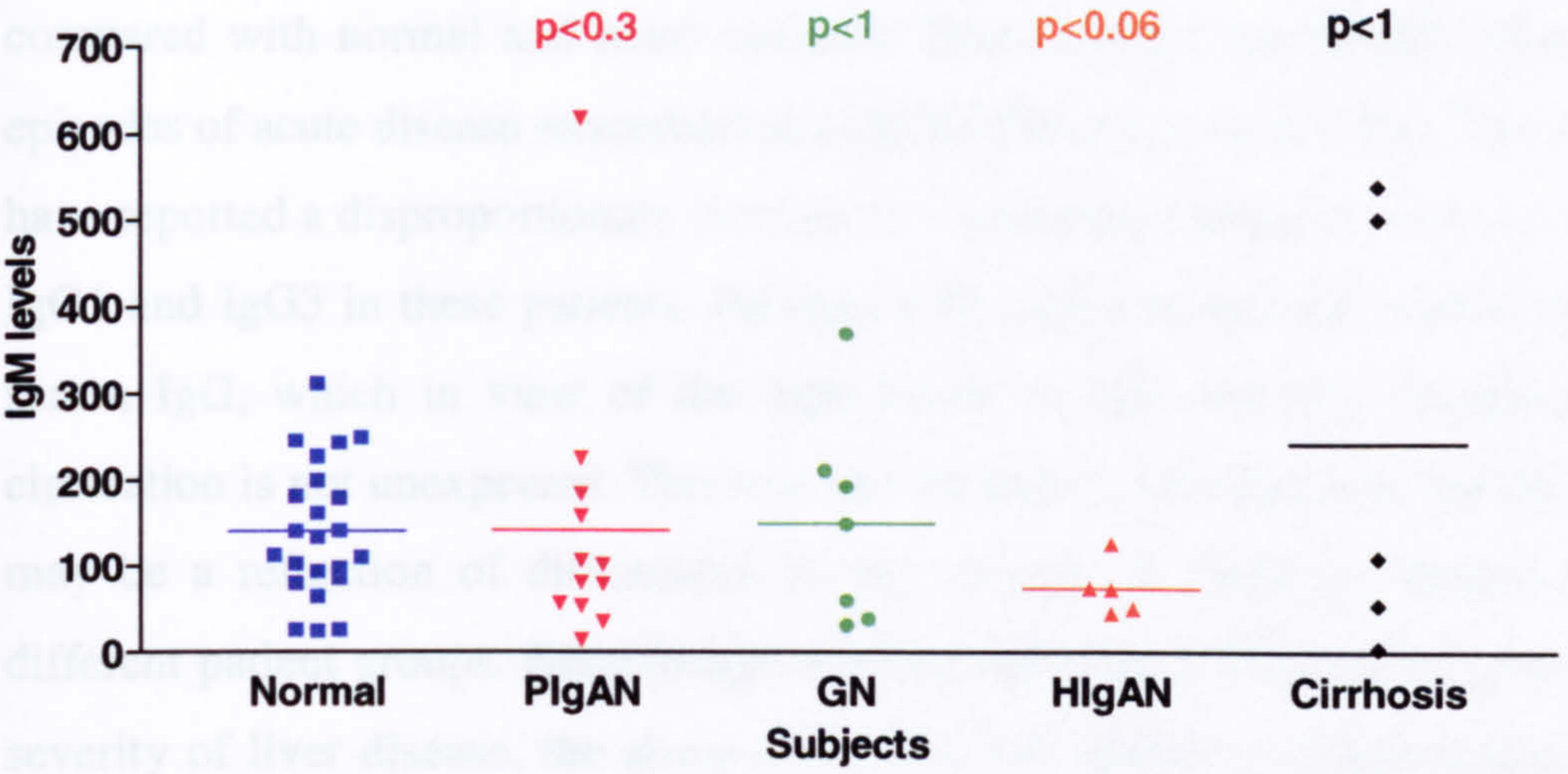



**Figure 7.12** IgM levels in supernatant produced by cells cultured in unstimulated RPMI medium. There were no significant differences in IgM production between the groups.

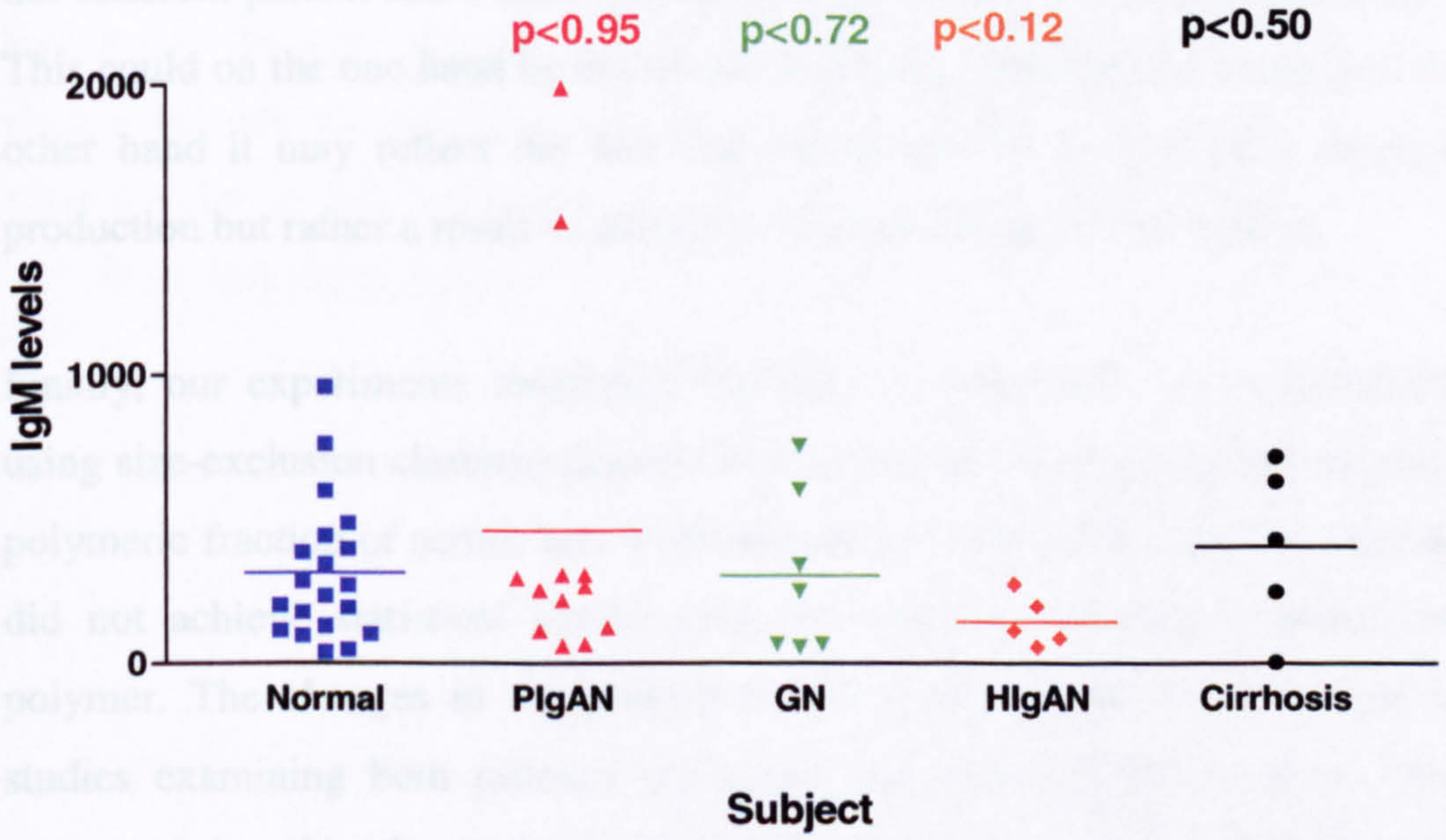



**Figure 7.13** Graph showing the IgM levels produced in the supernatant of PBMCs cultured in RPMI containing pokeweed mitogen. No statistically significant differences were found between the different groups.

Total serum IgG levels were found to be increased in patients with primary IgAN as compared with normal and renal controls. This has been previously reported during episodes of acute disease exacerbation in IgAN (Rostoker et al 1989). The same group have reported a disproportionate increase in circulating immune complexes containing IgG1 and IgG3 in these patients. Patients with cirrhosis showed marked elevation of serum IgG, which in view of the high levels of IgG immune complexes in their circulation is not unexpected. This was not the case in patients with hepatic IgAN and may be a reflection of differences in the severity of the liver disease in the two different patient groups. Even though we have attempted to match the two groups for severity of liver disease, the absence of a reliable marker of hepatic function makes this quite difficult.

Although not all observers agree, a number of previous studies have reported increased IgA production and IgA system hyperactivity *in vitro* studies of PBMCs cultured in stimulated and unstimulated medium. We did not find any differences in the different patient and control groups for any of the three immunoglobulin classes. This could on the one hand be due to our particular experimental technique, or on the other hand it may reflect the fact that raised IgA levels are not a result of over production but rather a result of defective clearance from the circulation.

Finally, our experiments measuring the ratio of polymeric versus monomeric IgA using size-exclusion chromatography confirms the previously reported increase in the polymeric fraction of serum IgA levels in patients with primary IgAN. Although this did not achieve statistical significance, the trend is definitely towards increased polymer. The changes in the polymeric IgA levels appear to be modest in some studies examining both primary IgAN and also cirrhosis of the liver. One study suggested that this elevation was a consequence of the overt increase in serum IgA levels and found that the monomeric IgA levels were also markedly elevated (Newkirk et al 1983). They did however find selective rise in polymeric IgA in alcoholic cirrhosis. Other studies have not found a marked increase in polymeric IgA in IgAN and in one study of antigen specific antibodies (Russell et al 1986) three patterns of predominant polymer, predominant monomer, and equal amounts of polymer and monomer were found. One group did find significantly higher polymeric IgA levels in IgAN versus patients with non-IgA renal disease and normal controls

(Tomino et al 1984). However the numbers of patients in each group were very small. Czerkinsky et al (1986) found that in a third of patients both monomeric and polymeric forms of IgA immune complexes were raised in IgAN.

The absence of a significant result in our study may also be due to less than optimal resolution of the various peaks obtained. The results none the less were highly significant in patients with cirrhosis and confirm the findings from previous reports of elevated polymeric IgA levels in this group. We would have expected patients with hepatic IgAN to show a similar trend but found no significant change as compared with the normal polymer to monomer ratio. This may be due to insufficient number of patients in this group.

### 7.4 Conclusion

In conclusion our results support the previous findings of elevated IgA levels in patients with primary and hepatic IgAN and cirrhosis. In addition we have found that this is also the case in patients with other types of glomerulonephritis. The PBMC studies did not support the previously reported hyperactivity of the IgA producing plasma cells in IgA nephropathy. Furthermore, raised IgA levels are in no way specific to IgA nephropathies when compared to the relevant disease controls.

# Chapter 8: Discussion

This thesis has examined the production, clearance and structural characteristics of human IgA in a number of IgA-mediated diseases. The main purpose of this study was to elucidate the mechanisms involved in IgA1 mesangial deposition choosing the IgA1 hinge region O-glycosylation pattern as its main focus. We believe no single aetiological factor is responsible for IgA deposition in IgAN and that several contributory factors are required for this diverse condition to develop in its various forms. An important strength of this study has been the use of samples from patients not only with primary IgAN, but also those with IgAN secondary to hepatic disease. Furthermore, appropriate renal and hepatic controls were employed which permitted the variations in both normals and other renal and hepatic disease groups to be determined.

The glycan content of the IgA1 hinge glycopeptide was determined using mass spectrometry. Having optimised the current MS techniques for performing O-glycosylation analyses, the spectra obtained were used to compare the glycan content of serum IgA1 in all patient and control groups. IgA production was assessed *in vitro* using PBMCs in culture and PBMC CD89 expression was studied by flow cytometry to assess one of its known clearance routes. Studying patients with secondary IgAN and patients with liver disease, has allowed us to examine a wide spectrum of conditions where IgA system anomalies have been identified, thus deepening our understanding of wider mechanisms underlying different subsets of patients in a disease which characteristically has a variable phenotype.

IgAN is recognised as the commonest type of glomerulonephritis in the Western world (Julian et al 1988). Since Berger and Hinglais first described IgAN in 1968, the condition has come to be known in a variety of heterogeneous clinical forms spanning a wide and disparate clinical spectrum (Nicholls et al 1985; Chauveau and Droz 1993). Such is the diversity of the clinical presentation, course, and outcome of IgAN in different individuals and populations that classing it all as one disease may be an over-simplification and highly questionable. The one feature that unifies these variable clinicopathological entities is the detection of mesangial IgA deposits on immunofluorescence associated with variable degrees of mesangial matrix deposition

and cellular proliferation (d'Amico 1988). A review of the literature covering the natural history of IgAN highlights the existence of extreme variability in both the clinical course and the rate of progression in patients labelled with this disease (d'Amico 2000). The risk factors that are associated with poor outcome and disease progression are not really specific to IgAN and are generic to most other types of renal disease. The conflicting and controversial results of hundreds of studies published claiming universal parameters for prognostication in this disease suggests that IgAN should be regarded more as a syndrome rather than a unified disease (Clarkson et al 1984). The majority of clinical studies of IgAN so far have primarily been in non-selective cohorts of patients (Koyoma et al 1997; Radford et al 1997; Nicholls et al 1984; d'Amico et al 1986). Perhaps future studies should focus on selected sub-groups with well-defined characteristics in order to highlight the nuances in the phenotypes of individuals in terms of their presentation, clinical course, risk factors for progression, and the underlying aetiology of IgA nephropathy in all its guises.

It is apparent from both clinical and laboratory studies of IgAN that there are as many permutations in the pathological and immunological profiles of these patients as there are in the clinical features (Emancipator and Lamm 1989). This, we believe is another strength of our study where we have assessed both primary and secondary forms of IgAN, describing the laboratory features of the individual types of the primary and hepatic forms of the disease as well as a number of cases of myeloma and HSP. This allows us to move away from an over-simplified 'one-size fits all' approach to the pathogenesis of such a heterogeneous syndrome.

The mechanisms of IgA deposition and the consequent initiation of glomerular injury in IgAN remain unknown and the unravelling of its aetiology continues to present us with a scientific challenge. Given that IgAN is distinguished from other mesangio-proliferative diseases by the presence of IgA deposits in the mesangium on immunofluorescence, one key to our understanding of the disease process must be a clearer appreciation of the factors leading to the deposition of IgA in the different forms of the disease. It follows that in studying these factors we appreciate the existence of different mechanisms leading to IgA deposition in different patients. The course the disease takes may to some extent be dictated by the individuals' genetic

predispositions to other factors such as hypertension or renal fibrosis. Elevated IgA levels and aberrant glycosylation of IgA1 in IgA nephropathies have been reported and may be the starting point in the development of the disease (Andre et al 1990; d'Amico 1988; Tomana et al 1997; Allen et al 1995; Mestecky et al 1997). They therefore warrant detailed investigation and characterisation in the process of unravelling the pathogenesis of IgA nephropathies.

Identification of the factors leading to IgA deposition and the initiation of the inflammatory process have been hampered both by the absence of good animal models of IgA disease (Allen 1999), general paucity of information on human IgA1 O-glycan structure due to inherent technical difficulties, and confusion and contradiction within the literature concerning various aspects of IgA metabolism (Allen 1999). The relative lack of detailed information on human IgA glycosylation has been in part due to the presence of a variety of glycoforms of IgA1 in any one individual as well as the complexity of the potential permutations of O-glycosylation in the hinge region (Reviewed in Novak et al 2001). This is changing, with increasing information becoming available from mass spectrometry as the technology has become more and more sophisticated and accessible, replacing less reliable techniques such as lectin binding and gas chromatography in the study of IgA structure (Odani et al 2000). Despite this, there are still many discrepancies in the literature regarding the mechanisms behind the production, clearance and physicochemical characteristics of IgA and the glycosylation pattern of the hinge region of IgA1. I will address some of the questions arising from these discrepancies in the light of our findings.

## 8.1 Summary of Key Findings

- Patients with primary IgAN have mass spectrometric evidence of significantly reduced serum IgA1 sialylation with a slight decrease in galactosylation as well as lower numbers of GalNAc residues.
- Patients with HIgAN also have marked undersialylation but increased galactose content compared with normal controls. Cirrhotics with no glomerular lesions have a greater proportion of undersialylated IgA1 in their circulation but the galactose and GalNAc content remains unaltered when compared with normal controls.

- The finding of altered glycosylation is not specific to renal diseases with IgA deposition. Patients with other glomerulonephritides also showed changes in their glycosylation pattern. Furthermore, when comparing the PIgAN and GN groups, no statistical differences were found between the two.
- Our patients with myeloma and HSP secondary to myeloma also showed changes in the IgA1 glycan composition.
- We found elevated total serum IgA levels in all the groups compared with normals. The finding of elevated total IgA levels in our renal (GN) control group was unexpected. IgA1 levels were elevated in primary and hepatic IgAN as well as patients with cirrhosis and non-IgA glomerulonephritis. IgA2 levels were high in patients with cirrhosis and GN. The ratio of polymeric to monomeric IgA was only increased in patients with cirrhosis without renal involvement.
- No significant changes were found in CD89 expression on peripheral blood mononuclear cells in any of the groups.

## 8.2 IgA1 O-Glycosylation

### 8.2.1 Glycosylation results

Previous attempts at obtaining spectra from the heavily sialylated IgA1 hinge glycopeptide has been met with variable success. The only group who has previously succeeded in isolating the hinge glycopeptide and studied it by mass spectrometry have been unable to obtain baseline resolved, discrete spectra that allowed quantitative analyses to be performed (**Figure 4.1**) (Iwase et al 1999; Hiki et al 1999; Odani et al 2000; Hiki et al 2001; Horie et al 2003). Their best spectra were obtained when the specimens were subjected to enzymatic cleavage of their sialic acid residues, thus producing spectra that were informative only about the galactose and GalNAc content of the peptide (Hiki et al 1998).

We have successfully established a methodology for assessing the intact IgA1 hinge glycopeptide using MALDI-ToF-MS. This allowed us to study the native IgA1 hinge region in detail without having to enzymatically desialylate the species under examination. This revealed a hitherto undiscovered 6$^{th}$ GalNAc substitution of the hinge glycopeptide in all patient and control samples (Tarelli et al 2004). The MS

results corroborated with the lectin binding data from studies performed on the same samples, confirming the robustness of our results (**Figures 5.18**).

When this technique was applied to the various IgA1 samples obtained from the sera of patient and control groups (including those with IgA myeloma and HSP secondary to IgA myeloma), a number of interesting structural features of the IgA1 HGP O-glycans became apparent (**Table 5.6**). The most striking changes in IgA1 O-glycosylation were in the degree of sialylation of the hinge region (**Tables 5.5** and **8.1**). Patients with primary and hepatic IgAN as well as those with cirrhosis were found to have a statistically significant reduction in the number of sialic acid residues in the hinge region. The most marked changes were in those patients with cirrhosis. Interestingly and rather unexpectedly, patients with non-IgA GN also showed, albeit to a lesser degree, evidence of under-sialylation but this was not statistically significant.

| | GalNAc | Galactose | Sialic acid |
|---|---|---|---|
| **Normal** | 4.52 | 3.6 | 2.42 |
| **PIgAN** | ↓ | ↓ | ↓↓ |
| **GN** | (↓) | ↔ | ↓ |
| **HIgAN** | ↓↓ | ↑ | ↓↓↓ |
| **Cirrhosis** | ↔ | ↔ | ↓↓↓ |
| **Myeloma** | ↓ | ↑ | (↓) |

**Table 8.1** A summary of glycosylation changes in the IgA1 HGP in the patient and control groups. The numbers represent the average glycan residues per IgA molecule calculated from the values obtained from the normal controls. ↑ elevated glycan content compare with normal, ↓ reduced glycan content compared with normal, ↔ unchanged.

A minor reduction in the percentage galactosylation of GalNAc residues on the HGP was observed which was only statistically significant in patients with primary IgA nephropathy (**Table 5.4**). Patients with hepatic IgAN in fact showed a slight increase in percentage galactosylation.

We discovered a 6$^{th}$ GalNAc substitution in between 5-10% of the HGP species in normals and all patient groups with the exception of those with myeloma. In addition, we discovered the presence of differences in the number of GalNAc substitutions between the groups (**Table 5.3**). Patients with primary and secondary IgAN and not their renal or hepatic controls had a larger proportion of glycoforms containing 3 GalNAc substitutions corresponding to a reduction in the mean GalNAc number. Those with myeloma and HSP myeloma had a trend towards higher mean numbers of GalNAc substitutions. There was no difference between mucosal or systemic IgA1 in this respect when comparing the glycan content of serum and breast milk IgA.

The most strikingly unusual O-glycans found in patients with PIgAN were found in the least abundant species. When presented in rank order, the data suggest that there is little difference in the ranking of the major (top 10) glycoforms between the primary IgAN and the normal and renal controls (**Table 5.5**). However a number of minor glycoforms were identified in the primary IgAN group which were totally absent in the controls. These were also absent from the renal controls (GN group) (**Table 5.7**). The significance of this finding is two-fold. Firstly it suggests that mesangial deposition of IgA1 may be mediated by a small but highly abnormal and pathogenic group of IgA1 glycoforms. Secondly, it raises the technical possibility that the most pathogenic glycoforms may not be retained by the jacalin affinity columns due to their highly unusual glycosylation and may be lost during sample purification and processing.

Our results do not support the findings of previous studies that have claimed reduced galactose content as the salient distinguishing feature of IgA1 O-glycosylation in primary IgAN based on lectin binding and HPLC techniques (Allen et al 1995, Tomana et al 1997). In one review article, the IgA HGP in IgAN has been firmly labelled as being 'Gal-deficient' quoting studies where the techniques used do not give as accurate or direct information about the structure of these glycans as mass

spectrometry (Novak et al 2001). Where they quoted the mass spectrometry data, they omitted to mention the findings pointing to de-sialylation of the HGP (Novak et al 2001). Our data presents further evidence of complex global changes in the glycan content and composition in the patients and controls we have studied. The significance of these findings is discussed below.

### 8.2.2 Interpretation of Glycosylation findings

IgA1 O-glycosylation is altered in both primary and hepatic IgANs but also in other non-IgA glomerulonephritides. Patients with IgA myeloma with and without the presence of renal disease also exhibit changes in their glycosylation as compared with normal but the pattern of change is distinct from those with IgA nephropathies and may reflect the particular clonal expansion unique to each case. This is the first report of alterations in the glycosylation of IgA1 in other GNs.

These findings could be interpreted in a number of ways. Firstly, altered glycosylation may be a symptom rather than a cause of IgA nephropathies and as such reduced IgA1 O-glycosylation may not be singularly implicated in their pathogenesis. The presence of altered IgA1 O-glycosylation in other renal diseases supports the hypothesis that these changes are not specific to IgAN per se. The glycan profile obtained for the different groups of patients and controls showed shifts in the preponderance of the various species rather than distinct changes in glycoform composition. In IgAN, the most commonly represented glycoforms were found to be almost identical to those of other GNs in terms of ranking and percentage of total peak area. We therefore cannot solely attribute the pathogenesis of IgANs to subtle shifts in the overall glycosylation pattern. Furthermore, there was no correlation between the degree of renal dysfunction and the degree of change in glycosylation (See **Figures 5.19** and **5.20**). This makes the presence of under-glycosylation an unlikely single pathogenic factor in the aetiology or natural history of the disease. Nevertheless, these findings do not rule out a role for glycosylation changes as a trigger in a chain of events leading to the development of renal inflammatory disease in a subset of patients.

The idea that IgA nephropathies are heterogeneous diseases with different aetiological factors may also explain these findings. The example of IgA system changes in

hepatic IgAN and also in patients with cirrhosis demonstrates that under-sialylation can be due to the failure of the IgA clearance mechanisms of the liver. This may lead to accumulation of de-sialylated IgA in the circulation and lead to tissue deposition but not necessarily renal disease. So IgAN, which is really a morphological diagnosis with variable presentation and natural history, may also have diverse pathogeneses.

Another interpretation of our results may be that the presence of specific pathogenic IgA1 glycoforms, such as those found in small quantities in patients with primary IgAN may be capable of triggering an inflammatory process once deposited in the mesangium of genetically susceptible individuals. These particular species were absent in normal controls as well as those with other GNs. These striking differences although quantitatively small may be aetiologically significant. It may be these quantitatively insignificant glycoforms that represent the pathogenic glycans with a higher propensity to mesangial deposition in this disease. Whether the low level is due to tissue deposition or due to losses during jacalin affinity chromatography is not clear. One may speculate that the most pathogenic glycoforms are so abnormal that their binding to jacalin is significantly altered and therefore lost in the process of purification. The good correlations between our lectin binding results on serum IgA and the spectra obtained from purified IgA1 mitigates against there being a technical problem with our methodology.

In one study Hiki et al (2001) used MALDI mass spectrometry to assess the glycosylation of IgA deposited in the mesangium of patients with primary IgAN. They were able to characterise some of the glycosylation features of the deposited IgA. Interestingly, they identified some of the unusual glycoforms that we specifically found in the serum of our patients with IgAN (the asialo-species 320, 400, and 420). This finding lends support to the hypothesis that these atypical glycoforms found in low quantities in the serum have a stronger affinity for the mesangium and may play a crucial role in the pathogenesis of IgAN. This also directs us towards the glycoforms to be studied in the future both in terms of their binding properties when isolated from serum and their ability to trigger an inflammatory response in the glomerulus.

The literature covering the various techniques in IgAN so far has suggested different glycan candidates for the pathogenic role in IgAN. Lectin binding studies in IgAN on

the whole have shown evidence of under-galactosylation (Ander et al 1990, Allen et al 1995, Baharaki et al 1996, Tomana et al 1997, Allen et al 2001). However a number of studies did not support these findings and in fact found the opposite to be true (Tomino et al 1995, Hiki et al 1996) and one paper contained conflicting results when different lectins were used within the same study (Allen et al 1995). A study by Hiki et al (1996) using gas-phase hydrazinolysis showed increased asialo-Galβ1,3GalNAc residues. Fluorofore assisted carbohydrate electrophoresis has demonstrated truncated IgA1 O-glycans with increased terminal GalNAc (Allen et al 1999). Mass spectrometry has provided more evidence for both under-galactosylation and under-sialylation. Some authors have concluded from work based on gas chromatography that under-galactosylation in IgAN is the consequence of over-sialylation and over-activity of the sialyl-transferases (Tomana et al 1997). None of the studies has suggested aberrant GalNAc substitutions.

So which glycan abnormalities are specific to IgAN and are they pathogenic? Our data confirm the earlier finding of under-galactosylation and under-sialylation of IgA1. Our findings highlighted a global decrease in glycosylation in all three residues particularly in sialic acid numbers. We have found differences in the number of GalNAc substitutions and a small but significant decrease in the percentage galactosylation. Given that patients with cirrhosis have strikingly under-sialylated IgA and very high serum concentrations of IgA1 with and without renal disease, it is unlikely that pure under-sialylation per se is the cause of progressive renal disease in hepatic or primary IgAN. This does not rule out the role of under-sialylated species in the initial formation of the deposits nor does it negate the potential role for specific under-glycosylated pathogenic glycoforms in the pathogenesis of the disease. I believe that the role of so-called 'Gal-deficient' HGP has been over-stated in the literature (Tomana 1997, Novak 2001) without much direct evidence for its existence in a sufficiently large cohort of patients.

How have the findings of this thesis contributed to our understanding of the pathogenesis of IgAN? Our study has analysed the greatest number of patients with IgAN and normal controls by mass spectrometry, engaging the appropriate normal and diseased controls. The real strength of the project has been in assessing defined

groups of patients with different characteristics and aetiologies using tools that inform us directly and accurately of the underlying structure of the IgA1 molecule. The study therefore has the power to draw definitive conclusions about the glycan content of IgA1 and its potential role in renal diseases.

The observation that IgA1 O-glycosylation alterations in themselves are not specific to IgAN, and in light of the discovery of changes in all 3 glycans, the stated importance of glycosylation per se in the pathogenesis of IgANs now needs to be reassessed. The presence of similar changes in glycoforms in diverse diseases invalidates automatic assumptions about the biological properties and the pathological role of O-glycans in the pathogenesis of IgANs even though they may have a share in its multi-factorial pathogenesis. The development and progression of the disease may require the interaction between a number of triggers and predisposing factors in any one individual.

One possibility is that the presence of a small number of highly pathogenic 'sticky' glycoforms, over a period of time in the circulation will lead to mesangial deposition and trigger inflammation. One may hypothesise that IgAN manifests itself when an excess of undersialylated glycoforms such as those found in the glomeruli of IgAN patients described by Hiki et al (2001) and those exclusively identified in the serum of patients with IgAN in this study are deposited in the renal mesangium. The progression of disease will depend on the genetic make-up of individual patients and their predisposition to other co-morbid factors such as hypertension. Perhaps different IgA1 glycoforms have variable inflammatory and pathogenic potential and the presentation of the patients depends on the mix of glycans present within the deposits (Kokubo et al 1999; Kokubo et al 2000). This could be supported by finding positive correlations between markers of progressive renal disease such as raised serum creatinine and the type of glycoforms encountered in the any one individual. We found no such correlation in our study.

Alternatively it is likely that the unusual IgA1 O-glycosylation is a consequence of the diseases we have studied. One can hypothesise that in patients with inflammatory diseases affecting the kidneys and liver, the metabolic and immunological changes resulting from cirrhosis or glomerulonephritis may affect immunoglobulin glycan

structure. Increasing evidence of glycosylation abnormalities in other autoimmune diseases such as rheumatoid arthritis may be a reflection of a universal change in post-translational modification of glycoproteins in an inflammatory milieu. The type of inflammatory process and the organs involved may dictate the exact nature of these changes.

Finally, in light of the variable descriptions of its clinical and morphological features, the natural history, as well as the contradictory findings regarding its aetiology by various researchers, IgAN probably needs to be viewed as a heterogeneous disease with a common histological appearance and not under the umbrella of a unified diagnosis. The literature derives its information from patients studied with diverse racial backgrounds from the Pacific Rim, through Europe to America. Therefore the different genetic influences should be taken into account when interpreting the findings of such studies. The cohorts studied are also not homogeneous with respect to the clinical features of the patients and present vast clinical diversity. It may be that the presence of a certain pathogenic subset of glycoforms in a cohort of patients with specific racial and genetic predispositions and characteristics are required for IgA nephropathy to manifest itself in a particular phenotype and with a course unique to itself. This hypothesis may be tested by isolating those aberrant forms we found exclusively in patients with IgAN and those found in the deposits of IgAN patients in the paper by Hiki et al (2001) and assessing their binding properties in vitro and in vivo to mesangial cells, their receptors, and extra cellular matrix components. However, any findings from such studies should be viewed in the context of the individual patients genetic, racial, and immunological characteristics.

### 8.2.3 Limitations of the MS study

The main significant limitation of our glycosylation studies are whether the results of experiments on serum IgA are truly a reflection of the tissue IgA within the renal mesangium. So far only two studies have addressed this issue (Allen et al 2001; Hiki et al 2001). Obtaining renal tissue from patients with IgAN for the elution of IgA is difficult. One study has eluted IgA1 from 3 nephrectomy samples and examined the glycosylation pattern by lectin binding whilst another eluted the IgA1 from renal biopsy samples and analysed them by MALDI ToF MS. The former found elevated lectin binding to Helix aspersa and Vicia villosa in all three tissue samples as

compared with the serum from the same patients and other patient and controls suggesting the presence of the under-sialylated and under-galactosylated forms in the glomeruli of these patients (Allen et al 2001). Hiki et al (2001) found decreased galactosylation and sialylation of the deposited IgA1 compared with serum IgA from IgAN patients and normal controls.

The concern about the use of jacalin in glycosylation studies and its implication in terms of selectivity for different glycoforms has been addressed earlier in chapter 5. Our results may be biased towards the least pathogenic glycoforms if there is an inherent inability of jacalin to bind the most aberrantly glycosylated species. However the concordance between our mass spectrometric findings and those of the lectin binding studies are reassuring that no significant glycoforms have been lost through the use of this method. The lectin binding assays were performed on whole serum not purified IgA1. The IgA was captured on anti-IgA coated plates before applying the lectins. These were done in duplicate plates that were developed with anti-IgA1 to ensure the wells were saturated with IgA1 and that the differences in lectin binding were not due to different amounts of IgA1 being present in the wells. The mass spectrometry was, on the other hand performed, on samples of purified IgA1. The fact that the two sets of studies and results show good correlation is highly significant because it suggests that the purified IgA1 on which the MS has been done is representative of the whole array of glycoforms in the sera and that there have been no major loss of glycoforms.

One further technical criticism may be directed at the differential ability of various glycoforms to 'fly' in the mass spectrometer vacuum chamber due to different masses. Thus for example under-sialylated species may be over represented as compared with more heavily glycosylated glycopeptides. Whilst the absolute percentages of the various glycoforms may be inaccurate, the differences between the groups should remain robust.

This study has provided detailed information about the overall carbohydrate moieties' numbers in the hinge region. However it does not provide any information about amino acid specific substitutions of the glycoforms and this kind of structural analysis will need to follow. The increasingly sophisticated mass spectrometry technologies

now available should allow the exact structure of the glycoproteins to be identified. With the expansion of the new MS techniques, there is much room for optimism for progress to be made in this area. Already Novak et al have presented exciting new results by identifying the sugar substituted amino acids in the tryptic fragments of the hinge region of an IgA1 myeloma protein. The desialylated spectra were obtained from a high resolution Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Novak et al 2004).

### 8.2.4 Future strategies in glycoform analysis

We are currently planning de-glycosylation studies to re-assess the galactose content of the hinge glycopeptide samples that have been already studied. The bulk of evidence in the literature that proposes IgA1 under-galactosylation is the hallmark of IgAN has not utilised mass spectrometry to assess its glycan content. We need to clarify this using our samples by a gold standard technique such as mass spectrometry. By removing heavy sialyl groups from the outer surface of the molecule, the core sugars and in particular actual galactose numbers will become more readily quantifiable. This should clarify the contribution of the different glycans in the make up of IgA1 in PIgAN.

Having described the relative carbohydrate content of the IgA1 HGP in detail, we would like to perform qualitative analyses of the amino acid positions where substitutions by glycans in the IgA1 hinge region have occurred. We now have access to the surface-enhanced laser desorption and ionisation (SELDI) MS technology, coupled with other facilities which should allow us to look at the exact structure of the molecule.

Whilst the one MS study of the renal mesangial deposits shed some light on the type of glycoforms in the kidney, technically it did not produce optimal spectra for performing a definitive analysis of the deposits (Hiki et al 2001). We would therefore like to repeat this study using nanotechnologies that require the minimum amount of sample to assess IgA1 glycoforms eluted from renal biopsy samples. It is now possible using single glomerular isolation techniques to perform such studies without requiring large quantities of biopsy tissue. Once specific deposited glycan changes are identified, it may then be possible to delve into the cause of the specific glycosylation

changes. The study of glycoform profiles for both serum and mesangial IgA1 could reveal more information about the role of IgA1 O-glycosylation in this disease. Furthermore, the chemical properties of the under-glycosylated glycoforms could be compared with the normal controls. A number of studies described earlier have looked at the binding properties of de-sialylated IgA on columns lined with ECM and also their deposition when injected into experimental animals. Further analyses of the properties of glycoforms presumed to be pathogenic and found in the sera and mesangium of IgAN will be desirable. Likewise, the ability of these glycoforms to trigger an immune response will be of interest.

Another interesting aspect of the glycosylation of IgA about which very little is known is the mechanism and site of glycosylation. Information on the factors controlling the number and order of glycan substitutions on a particular glycoform is scanty. Unravelling these processes is not only of academic interest but also opens potential routes for therapeutic interventions to alter glycosylation in the future.

## 8.3 IgA production and sub-class profile

### 8.3.1 Findings of the studies

Serum IgA levels were found to be elevated in all study groups as compared with normal controls. Whilst this confirmed previous findings in patients with primary and hepatic IgAN as well as those with alcoholic cirrhosis, it was an unexpected finding in patients with glomerulonephritis of non-IgA aetiology. IgA1 levels were also elevated in patients with IgAN, HIgAN and cirrhosis and again in patients with other GN. The IgA2 levels however were only significantly raised in patients with cirrhosis and rather unexpectedly again in GN. In keeping with previous findings of changes in the IgG immune system, serum IgG levels were also found to be higher in patients with IgAN and cirrhosis compared with normal controls and other GN (Schena et al 1986; Giron et al 1992).

The ratio of polymeric to monomeric IgA was increased significantly in patients with cirrhosis as compared with normal controls. In IgAN, although the trend was towards the presence of higher polymer forms of IgA, this change in ratio did not reach statistical significance compared with normals and renal controls.

Despite elevated levels of total serum IgA in all our patient groups and diseased controls, no differences in IgA production by PBMCs in culture, neither in the resting nor stimulated state, were found. Given that the stimulated cultures produced higher levels of immunoglobulins of all three (IgG, A, and M) classes compared with the unstimulated group, it is unlikely that the lack of differences between the groups is due to an experimental artefact. These findings bring the significance of IgA levels as a marker of disease under question. Furthermore, the presence of elevated IgA levels in renal diseases not mediated by IgA makes serum total IgA levels an unreliable diagnostic marker.

IgA production *in vitro* is sensitive to many different culture conditions that will inevitably vary from one investigation to another. The fact that many researchers report conflicting results regarding IgA hyperactivity *in vitro* may be down to lack of uniform culture conditions used in different studies (Toyabe et al 2001; Layward et al 1994; Hale et al 1986; Egido et al 1983; Linne and Wasserman 1985; Feehally et al 1986; Cagnoli et al 1985). The artificial tissue culture environment and lack of normal intercellular signalling pathways may also affect the growth and development of PBMCs *in vitro*. The choice of PWM may not reflect the true *in vivo* stimuli that trigger inappropriate production of IgA by natural food or infective antigens (Russell et al 1986). Our findings therefore do not rule out *in vivo* IgA over-production.

We cannot draw definitive conclusions from the slight but statistically non-significant predominance of polymeric IgA over monomeric IgA in the serum of our patients with IgAN. Our findings support other research where absolute levels of serum polymeric IgA were increased, but levels of polymeric IgA relative to total serum IgA were in fact lower in IgAN when compared with normal controls (van der Boog et al 2004). This study also found no significant correlation between serum concentrations of polymeric IgA and clinical parameters of disease. These data support the notion that size alone is not the key factor for mesangial deposition, while the physicochemical composition of the macromolecular IgA may be.

### 8.3.2 Limitations of IgA levels and production studies

As with any clinical study, the patient groups may not be necessarily uniform in terms of degree of renal or hepatic dysfunction. Every effort was made to select patients with near normal renal function. However a number of patients with renal failure were also included in the study. A number of clinical parameters for each group of patients were observed, such as serum creatinine levels, γGT levels, urine dipstik analysis, and 24-hour protein levels. Assessment of hepatic function is more challenging and again as far as possible, patients with cirrhosis did not have decompensated liver failure. Although none of our patients were on immunosuppression, matching was not possible for other medications and most patients were on anti-hypertensive drugs and ACE inhibitors. It was not possible to match the study groups for gender due to preponderance of males in the IgAN and HIgAN patients. One further clinical criticism involves the study being carried out at a single time point. Whilst every effort was made to rule out acute infections or inflammation at the time of recruitment, it is difficult to generalise the results from the study at a single point in the natural history of the disease. Ideally one would want to repeat these studies over a period of time to look for changes with ageing and with acute inflammatory episodes.

Finally, at the onset of this project, we assumed, based on the current literature, that patients with hepatic IgAN generally have a more benign course and do not develop renal failure. They would therefore act as a control group with a good prognosis for patients with primary IgAN. The literature suggests that ESRF in HIgAN is exceedingly rare. In our unit, out of 8 cases that were identified with HIgAN, 4 developed end-stage renal disease, and another 2 had severe chronic renal failure. One had mild renal failure with only one case with normal renal function after a 4 year follow up period. In effect, we did not have a 'benign control group' as had been anticipated at the outset. This does, however, suggest that HIgAN is not as innocuous a renal condition as it has been supposed. We may have selected the most aggressive cases by virtue of the fact that we only included patients if they had biopsy proven HIgAN. Given that patients without significant renal impairment would not have been biopsied, our sample may not truly represent the HIgAN population.

## 8.4 IgA clearance mechanisms

We did not demonstrate any statistically significant differences in CD89 receptor expression on either monocytes or neutrophils in any of the study groups. The percentage of CD89 positive neutrophils and monocytes were also found to be uniform between the patient and control groups. This did not appear to be a reflection of differences in technique or the state of activation of the cells. It is unlikely that in IgAN, altered CD89 expression per se is a significant reason for a potential defect in the clearance of IgA and its immune complexes. This does not rule out the possibility of defective clearance due to altered interaction between the ligand and its receptor. As more and more Fcα receptors are identified, it may be that other clearance mechanisms within the reticulo-endothelial system attain greater significance and studying such routes may prove more instructive.

In patients with hepatic cirrhosis, it is more likely that the cirrhotic liver and the damaged hepatocytes are unable to perform their normal clearance of IgA1 macromolecules and that this leads to IgA accumulation within the circulation. One study by Burgess et al (1992) showed that the changes in polarity in cirrhotic hepatocytes altered the position of the ASGP-R. This may in turn affect the accessibility of IgA to the receptor for clearance. Further studies of these disease specific mechanisms in these patients are warranted to assess the structure and function of these receptors and their interactions with their ligand using liver biopsy material.

Finally, we are interested in studying the role of hepatocyte clearance of IgA and the interactions between glycan structure and the ASGP-R. This may be done either using immortalised cell lines such as the HepG2 that express the ASGP-R or using liver biopsy material from patients with primary and hepatic IgAN.

## 8.5 Final Remarks

This project aimed to study IgA biology as relevant to IgAN. In order to test the hypothesis that IgA1 O-glycosylation plays a role in the pathogenesis of IgAN, we analysed the various glycoforms of IgA1 by devising a refined technique for the HGP purification and analysis by MALDI. This allowed us to successfully identify a

myriad glycoforms, which represent the normal spectrum of permutations in the IgA1 HGP O-glycans, obtained from a large sample of normal volunteers. We subsequently compared different patient and control groups with this normal group using a Java programme created for this purpose. We also aimed to study the characteristics of serum IgA which we performed by measuring total IgA, IgA1, and IgA2 as well as the ratio of polymeric to monomeric IgA. IgA metabolism was also examined by looking for any differences in IgA production by plasma cells *in vitro* as well as IgA clearance via the CD89 receptor by measuring CD89 expression on peripheral blood mononuclear cells.

The major contribution of this thesis to our understanding of the pathogenesis of IgA nephropathies is in our mass spectrometry findings of IgA1 structure. We accurately identified and presented the vastly heterogeneous glycoforms that form the bulk of normal and diseased IgA1. Normal IgA1 glycosylation has not been previously assessed in such large numbers of volunteers. We have thus created a baseline for normal IgA1 glycosylation as well as defining the changes in the other patient and control groups. We also successfully compared the groups and found significant differences in both the core and surface glycans of IgA1. Our findings have proven the glycosylation changes in the IgA1 molecule as a global phenomenon in the populations we studied and as such not specific to IgAN. We did however identify certain highly abnormal glycoforms which were specific to patients with IgAN which warrant a closer examination as outlined above. We also confirmed the previous reports of IgA1 undersialylation in IgAN and to a lesser degree under-galactosylation. Our findings do not suggest that galactose-deficient IgA HGP is the most characteristic glycoform present in IgAN. By studying different subsets of IgA mediated diseases, different glycan patterns emerged in each group. These patterns may account for the variable presentation, clinical course and final outcome in the different groups. Studying the different IgA-mediated diseases as separate groups has allowed for these subtle changes to become apparent whereas examining them under a unified category would have blurred the differences observed.

In reviewing the IgAN literature on IgA levels and metabolism, we have come across much confusion and controversy. In this regard, our study confirms that elevated total serum IgA and its subtypes are not specific to IgAN. In patients with other GN the

IgA2 levels were in fact higher than IgAN. These serum levels should not have a place as a marker for the diagnosis or progression of the disease. Our data do not support the view held by some researchers in this field that polymeric IgA is the predominant form present in the circulation of patients with IgAN. We can only say that in a proportion of patients there is an increase in the polymer to monomer ratio. Furthermore our results show that there is no intrinsic over-production of IgA *in vitro* and an alternative mechanism for elevated IgA levels should be pursued. Finally we showed quite clearly that monocyte and neutrophil CD89 expression is unaltered in IgAN and we cannot attribute raised IgA levels in any of the patient or control groups to decreased clearance via reduced CD89 expression.

# Chapter 9: References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